MINIREVIEW

Effects of inoculation with plant growth-promoting rhizobacteria on resident rhizosphere microorganisms

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are exogenous bacteria introduced into agricultural ecosystems that act positively upon plant development. However, amendment reproducibility as well as the potential effects of inoculation upon plant root-associated microbial communities can be sources of concern. To address these questions, an understanding of mutual interactions between inoculants and resident rhizosphere microorganisms is required. Mechanisms used by PGPR can be direct or indirect; the former entails the secretion of growth regulators and the latter occurs through the production of antimicrobial compounds that reduce the deleterious effects of phytopathogens. The different modes of action may lead to different relationships between an inoculant and root microbial communities.

Introduction

Environmentally friendly biotechnological approaches offer alternatives to chemical control of plant diseases and pests, and to the application of chemical fertilizers (Dobbelaere et al., 2003). Among these alternatives, the development of plant growth-promoting rhizobacteria (PGPR) inoculants is exemplary and their use is constantly increasing. For example, there are now close to 25 million hectares of soybeans inoculated with *Bradyrhizobium japonicum* in South America and close to 500 000 hectares of wheat and maize inoculated with commercial *Azospirillum* inoculants in Argentina and Mexico (Fuentes-Ramirez & Caballero-Melindo, 2005; Izaguirre-Mayoral et al., 2007; Diaz-Zorita, pers. commun.) (Table 1).

Most PGPR are active when close to or in contact with the root of the inoculated plant, i.e. in the rhizosphere, a compartment in the soil matrix. Most rhizobacterial communities are drawn from the soil and fewer originate from seed-associated microorganisms but all are selected for growth in the rhizosphere, reaching a high density in that milieu (Curl & Truelove, 1986). The rhizosphere is a complex habitat: there, the action of a growing root responding to its environment combines with that of the biotic (mostly the resident microorganisms) and abiotic soil components, which also respond to their environments. The introduction of a large amount of exogenous bacteria as an inoculant has the potential to affect these resident microorganisms, and similarly, an inoculant may be affected by them. Such interferences may result in increased, decreased or no effect on PGPR effectiveness and from there developed the need to study the microbial ecology of the rhizosphere following PGPR applications.

Mechanisms of plant growth promotion (PGP) may be direct or indirect. Direct PGP entails the secretion of plant growth regulators like phytohormones that elicit root metabolic activities or the supply of root-derived nutrients (Burdman et al., 2000). Indirect PGP occurs when PGPR lessen or prevent the deleterious effects of phytopathogens through the production of antimicrobial compounds, competition for iron and nutrients or for colonization sites, to mention a few mechanisms (Whipps, 2001). This is not to say that a PGPR exclusively belongs to one of the two classes. It may also be endowed with both capabilities...
(Fuentes-Ramirez & Caballero-Mellado, 2005). These different modes of action may lead to different relationships between an inoculant and root microbial communities. Understanding of these mutual influences is now possible, due to the availability of new analytical and computational methods.

**Stresses affect the structure of soil microbial communities**

It is easier to observe effects on the output when the input is largely altered. External, extrinsic factors applied to soils may be severe, such as when soil becomes contaminated with heavy metal or with volatile aromatic hydrocarbons that decrease both the biomass and diversity of the bacterial community (Griffiths et al., 2000; Gans et al., 2005; Girvan et al., 2005; Li et al., 2006; Yao et al., 2006). Such pressures can potentially impact upon soil quality and productivity and lessen its stability when challenged with environmental fluctuations (Girvan et al., 2005).

Other hard stresses like desiccation, salinity and temperature change affect microbial population structure. Exposure to extreme environmental conditions as imposed during a dry season or by climatic changes altered soil microbial activity from under the canopy of *Tamarix aphylla* in a desert and from under oak cover in a Mediterranean ecosystem, respectively (Waldrop & Firestone, 2006; Shamir & Steinberger, 2007). In another example of drastic change, a 70% increase in atmospheric CO₂ concentration led to a shift in the community composition of *Rhizobium leguminosarum* bv. *trifolii*, affecting the competitiveness of strains in a pasture ecosystem (Montealegre et al., 2000).

These examples may be seen as extreme stresses. Yet, it is do not known whether they lead to transient or to permanent alterations in community structure. What is a catastrophic event or a perturbation, or a disturbance for such a complex community? For example, do seasonal or other cyclic systems really ‘rebound’? (Smalla et al., 2001).

**Agricultural practices, ecological disturbances**

Agricultural practices may not seem as severe, but they also lead to large alterations in soil parameters that may affect the rhizosphere. Physical changes in soil structure after tillage (Lupwayi et al., 1998), crop rotation (Alvey et al., 2003), and wastewater irrigation (Oved et al., 2001) were shown to impact upon soil and root-associated bacteria. Crop rotation increased bacterial diversity in the rhizosphere (Lupwayi et al., 1998; Alvey et al., 2003). Nevertheless, continuous grassland sustained more diverse soil bacteria than rotation of cereals and potato (van Elsas et al., 2002). Tillage reduced the diversity of bacteria, but this reduction was more prominent in bulk soil than in the rhizosphere (Lupwayi et al., 1998). A similar response was detected when compost was applied to soil (Inbar et al., 2005). Only when high levels of compost were applied were compost-derived species detected on the root surface (Inbar et al., 2005). Strong selection in the

<table>
<thead>
<tr>
<th>Country</th>
<th>Crop and bacterial species</th>
<th>Inoculated area</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>Soybeans – <em>Glycine max</em> <em>Bradyrhizobium japonicum</em></td>
<td>~10 million ha</td>
<td>65% of the soybean crop is inoculated</td>
<td>Ferraris et al. (2006); Izaguirre-Mayoral et al. (2007); Martin Diaz-Zorita (pers. commun.)</td>
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<td></td>
<td>Wheat – <em>Triticum aestivum</em> <em>Bradyrhizobium japonicum</em></td>
<td>~140 000 ha</td>
<td>~3.0% of the planted area</td>
<td>Martin Diaz-Zorita (pers. commun.)</td>
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<td></td>
<td>Maize – <em>Zea mays</em> <em>Azospirillum brasilense</em></td>
<td>~9000 ha</td>
<td>Increased interest</td>
<td></td>
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<tr>
<td>Brazil</td>
<td>Soybeans – <em>Bradyrhizobium japonicum</em></td>
<td>~13 million ha 500 000 ha</td>
<td>75% of the soybean crop is inoculated 11% of the bean crop (4.5 million ha) is inoculated</td>
<td>Ferraris et al. (2006); Izaguirre-Mayoral et al. (2007)</td>
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<td></td>
<td>Beans – <em>Phaseolus vulgaris</em> <em>Rhizobium tropici</em></td>
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<tr>
<td>Bolivia</td>
<td>Soybeans – <em>Bradyrhizobium japonicum</em></td>
<td>400 000 ha</td>
<td>60% of the soybean crop is inoculated</td>
<td>Izaguirre-Mayoral et al. (2007)</td>
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<tr>
<td>Uruguay</td>
<td>Soybeans – <em>Bradyrhizobium japonicum</em></td>
<td>380 000 ha 600 000 ha</td>
<td>100% of the soybean crop is inoculated 15% with liquid inoculants</td>
<td>Izaguirre-Mayoral et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Forage legumes Different rhizobia species.</td>
<td></td>
<td></td>
<td>Carlos Labandera (pers. commun.)</td>
</tr>
<tr>
<td>Mexico</td>
<td>Maize – <em>Azospirillum brasilense</em></td>
<td>60 000 ha 350 000 ha</td>
<td>2006 predicted for 2007</td>
<td>Jesus Caballero-Mellado (pers. commun.)</td>
</tr>
</tbody>
</table>

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rhizosphere therefore seems to mitigate disturbances occurring in the bulk soil. While soil type definitely can affect root-associated microbial community structure (Seldin et al., 1998; Dalmastri et al., 1999), it was reported that three plant species had different rhizosphere communities when grown in sand or in loam, but these distinctions waned in two of the three species when they were grown in a heavier clay soil (Marschner et al., 2004). Such effects should therefore not be neglected when PGPR are applied.

Plant factors affecting root-associated bacteria

A simple representation would be that edaphic and plant parameters apply ‘opposing’ pressures and whichever is stronger ‘wins’. Plant factors that have an influence upon microbial communities include plant age (Hamlen et al., 1972; di Cello et al., 1997; Natsch et al., 1998; Herschkovitz et al., 2005a,b) (Fig. 1a and b), plant species or even plant genotype (Latour et al., 1996; Dalmastri et al., 1999) and root exudates (Neumann & Römheld, 2000; de Weert et al., 2002). It should be noted that among root-associated populations, some may be more sensitive to a change in exudates (as may occur during plant growth) than others (Herschkovitz et al., 2005b) (Fig. 2).

Biological processes in the rhizosphere are strongly influenced by plant root exudates, which consist of easily degradable organic carbon compounds that attract and stimulate microbial growth (Lynch, 1990). The most pronounced aspect of this ‘rhizosphere effect’ is quantitative,
Fig. 2. Denaturing gradient gel electrophoresis (a–c) and dendograms of the PCR-DGGE profiles obtained with group specific primers (d–f) of rhizosphere (lanes 1, 2, 7, 8) and rhizoplane (tips – lanes 3, 4, 9, 10; branching sites – lanes 5, 6, 11, 12) of control (odd-numbered lanes) and of inoculated with *Azospirillum brasilense* strain Cd (even-numbered lanes) maize plants from the first (lanes 1–6) and third (lanes 7–12; 7–11 for *Bdellovibrio*) weeks post emergence. (a, c) *Alphaproteobacteria*; (b, d) *Actinobacteria*; (c, e) *Bdellovibrio* spp. No effect of inoculation with *A. brasilense* was detected. *Alphaproteobacteria* and the predatory bacteria *Bdellovibrio* spp. are affected by plant age while the actinobacterial population structure remains unaffected. From Herschkovitz et al. (2005b) (by permission).
with microbial population sizes and activities increasing closer to the root (Curl & Truelove, 1986, Fig. 1c and d). Finer resolution of the spatial component reveals that the distribution of microbial populations can be affected by location in the root system (Schallmach et al., 2000; Yang et al., 2001; Marschner et al., 2004). Nonetheless, it can be difficult to disentangle what to whom as root-associated microorganisms alter root exudation. For example, flavonoids, whose role in rhizobia–legume interactions is well described, may further act to shape the rhizosphere microbial community’s structure by acting on, and by being altered by ‘nontarget’ rhizobacteria (Shaw et al., 2006).

In the bulk soil population sizes were larger, but in the rhizosphere the phylogenetic diversity is more restricted (Marilley et al., 1998; Marilley & Aragno, 1999; Berg et al., 2005). The high concentration of easily metabolizable resources (exudates) in the rhizosphere sustains microbial populations that are more active, denser but less diverse than those present in bulk soil (Inbar et al., 2005). Diversity is further diminished in the rhizoplane at the plant–soil interface (Mavingui et al., 1992; Seldin et al., 1998; Herschkovitz et al., 2005a). Although more seldom addressed, soil physical parameters (the soil matrix) are also considerably altered by exudation (Walker et al., 2003) and may further select for rhizosphere-competent traits.

It is obvious that reciprocal interactions between soil, plant and microorganisms occur and that they are complex and should be accounted for (Walker et al., 2003; Marschner et al., 2004; Costa et al., 2006). However complex this is to achieve, beneficial outcomes of understanding such trilateral interactions on rhizosphere communities may be reaped when data become available. For example, in a study by Berg et al. (2005, 2006), it was shown that naturally occurring rhizobacterial and fungal communities antagonistic to Verticillium dahliae were further enhanced in certain plant species and at determined sites of cultivation.

In summary, current data show that while the microbial diversity may appear labile, cause and effect relationships linking community structure to environmental, biological and agrotechnical parameters are only just emerging. This will be very important knowledge when applied to microbial inoculants on a large scale.

**Effects of PGPR inoculation on resident bacteria**

Modifications in the soil–plant–microorganism partnership bring about intricate reaction mechanisms. What happens to rhizosphere microorganisms when a PGPR is introduced at high levels in the rhizosphere? Unsurprisingly, the answer is: it depends. This dependence may stem from interactions within and between indigenous populations. In relation to the soil–plant–environment background, certain groups may be enhanced, while others may be inhibited, or the introduced PGPR may not affect population structure (Nacamulli et al., 1997; Schwieger & Tebbe, 2000; Bacillo-Jimenez et al., 2001; Dobbelzaere et al., 2003).

PGP leads to more roots in the soil volume, as was shown in maize inoculated with Azospirillum brasilense (Dobbelzaere et al., 2003). PGP by this bacterium appears to result from a direct mechanism based on phytohormone excretion. In this system, neither dominant nor specific bacterial populations detected by PCR-denaturing gradient gel electrophoresis (DGGE) (a fingerprinting technique based on the detection of polymorphism in a marker gene common to the targeted populations) with general and group-specific primers, respectively, were affected upon inoculation (Figs 1 and 2) but the plants developed a denser root system (Herschkovitz et al., 2005a,b; Lerner et al., 2006). Inoculation with such PGPR can still affect the environment: as a consequence of higher microbial densities and higher metabolic (enzymatic) activity C, P and N turnover is increased in the rhizosphere (Mawdsley & Burns, 1994; Johansen & Binnerup, 2002). A more developed root system means a higher rhizosphere/bulk soil ratio and thus a more active soil, a characteristic thought to be positive (Winding, 2004).

A logical hypothesis is that indirectly acting PGPR such as antibiotic compound-secreting bacteria alter rhizobacterial communities. However, colonization of the rhizosphere of various plants with 2,4-diacetylphoroglucinol, phenazine or other antibiotic-producing fluorescent pseudomonads only had temporally and spatially limited and transient effects on rhizobacterial or fungal populations (De Leij et al., 1998; Herschkovitz et al., 1997; Girlanda et al., 2001; Moënné-Loccoz et al., 2003). PGP by this bacterium appears to result from a direct mechanism based on phytohormone excretion (Girlanda et al., 2001; Naseby & Lynch, 2001; Bakker et al., 2002; and see section on ‘genetic manipulations of microorganisms and plants’). Even when soils were systematically replanted in cycling experiments, the overall reported effects were small (Landa et al., 2003; Blouin-Bankhead et al., 2004).

Although large fractions of isolated rhizobacteria may be sensitive to the antibiotic produced by the PGPR *in vitro*, these populations are seldom affected by the presence of the inoculated bacteria *in vivo*, even under gnotobiotic conditions (Natsch et al., 1998; Johansen & Binnerup, 2002). Niche overlap between an inoculant and resident bacteria appears to be limited, even with resident organisms that are phylogenetically closely related to the inoculant. Spatial separation and nutrient versatility are certainly important dimensions contributing to this restricted overlap.

Nevertheless, some studies do point to longer-term residual effects of antibiotic-producing PGPR on resident bacteria such as when 2,4-diacetylphoroglucinol production by *Pseudomonas fluorescens* F113Rif appeared to cause a reduction in rhizobial diversity (Walsh et al., 2003). Other studies point to strong shifts in the community structure of some specific bacterial groups. For example, the production...
of trifolitoxin by *Rhizobium etli* strongly reduced the proportion of trifolitoxin-sensitive bacteria of the Alphaproteobacteria (Robleto et al., 1998).

While the results of most studies suggest that other factors, such as the ones mentioned in the previous sections, may be more determining in shaping rhizobacterial community structure when an exogenous, active PGPR introduced at high levels, this conclusion should be considered with some reservation: (1) Very few long-term studies have been conducted in which PGPR were systematically introduced and their effects measured. (2) Knowledge of the actual mechanisms responsible for the PGP effect that are actively deployed *in situ* by the introduced microorganisms are scarce. (3) The definition of adjectives such as ‘transient’ and ‘limited’ is as fine as the resolution applied in the study and what is accepted by society. Moreover, such transient or limited effects on community structure may still have unknown functional effects.

### Genetic manipulations of microorganisms and plants – impact on resident bacteria

Public sensitivity over environmental risks is rapidly growing, encompassing agricultural practices and other aspects of food production. The most sensitive issue is that of the genetically modified organisms, including microorganisms (GMMs). Ecological perturbations in microbial communities brought about by massive applications of PGPR appear to be small (after all, most of today’s agriculture is the massive introduction of a single species in a limited area, a catastrophic activity for natural ecosystems), and such practices usually do not generate antagonism, in sharp contrast to the reactions elicited by the use of, or even research on genetically modified (GM) organisms. Whether GMMs based on established PGPR have a stronger impact on microbial communities than their wild-type counterparts or not is a legitimate question validated by the potential of GMMs to persist for long periods.

In the sections above, it was observed that wild-type strains (GMMs that ‘only’ bear tractable markers and were shown to act as wild type were included) mostly had little or no detectable effect on resident bacteria, while other factors played principal roles in shaping community structure. Yet, one can hypothesize that an organism engineered to produce elevated levels of compounds proven to affect PGPR performance may ‘tip the balance’ and become one such factor that does have an impact upon resident populations.

The results of inoculation trials of soils or plants with antibiotic-secreting GMMs in a number of different crop systems were in most cases not significantly different from those generated by the introduction of the cognate wild-type strains.

Glandorf et al. (2001) reported field experiments in which *Pseudomonas putida* strains overexpressing phenazine genes exerted significantly longer antifungal effects than the wild-type strain. However, in subsequent trials, with repeated applications of 2,4-diacetylphoroglucinol and phenazine-producing GMMs, ascomycete populations were not affected more in the long-term than were those exposed to the wild-type *Pseudomonas putida* strain (Viebahn et al., 2003, 2005). A number of other studies showed that the rhizobacterial communities did not experience long term disturbances when exposed to increased dosages of antibiotic-producing genes through the introduction of GMMs (Natsch et al., 1997, 1998; Naseby & Lynch, 2001; Bakker et al., 2002; Bankhead et al., 2004). In an interesting study by van Dillewijn et al. (2001), the introduction of a proline dehydrogenase gene (i.e. a metabolic gene) into *Sinorhizobium meliloti* enhanced the latter's fitness. This enhanced strain with increased competitiveness only slightly affected the microbial community structure (van Dillewijn et al., 2002), suggesting that the different classes of genes (encoding for antibiotic production or for metabolic functions) hitherto used in GMMs exert limited effects on their microbial surroundings or on nontargeted organisms. As in previous examples, other factors were more significant in influencing rhizosphere microbial community structure.

GMMs may be ‘tough to sell’ but most of the fears and action concentrate on genetically modified plants and their potential as environmental hazards. Here, the focus is only on the possible impact of GM plants on the rhizosphere. As genetic engineering allows the formation of plants that differ by only one or two genes, the question whether these genetic differences are enough to influence the biodiversity of rhizosphere microorganisms associated with these genetically modified plants has been raised (Dunfield & Gemida, 2004). One way GM crops may alter the rhizosphere may be through natural transformation of soil microbiota with the introduced genes. However, while transgenic plant DNA persists in soil, the transformation of plant DNA to soil microorganisms has not been found (Dunfield & Gemida, 2004).

It can be assumed that impacts on rhizosphere microbial communities can result from two types of effects of transgenic products: (1) Secondary effects due to the presence of an ‘unrelated’ transgene on microbial systems through the release of novel compounds into the environment. These compounds may not necessarily be novel but either they are new in the rhizosphere, or they are not naturally found at the same level as that resulting from the presence of an engineered plant alone. These are unintentional effects. (2) Transgenes intended to affect or to manipulate rhizosphere populations. These are deliberate actions. This latter group may be further divided into manipulations aimed at reducing targeted populations, and those aimed at enhancing chosen microbial groups.
The first type (intended effects) constitutes practically all of the commercialized transgenic plants, with weed-resistant and pest-resistant crops (soybean, maize, canola, cotton and alfalfa) accounting for 68% and 19%, respectively, of the c. 102 million hectares planted in 2006. The remaining 13% of the land was planted with plants transformed for both traits (James, 2006).

Sensibly, most of the research has been performed on Bt and herbicide-expressing plants. A Bt toxin (the Cry protein) used to combat insect pests and introduced into maize had no effect on rhizosphere biomass and on the culturable fractions of bacteria and of fungi in the rhizosphere. Microfauna also appeared to be indifferent to it (Saxena & Stotzky, 2001). Further work revealed that different transgenic Bt lines acted differently upon soil microorganisms, and that the Bt toxin itself did not alter culturable bacterial or fungal root communities, although the Cry protein is excreted from transgenic plant roots (Donegan et al., 1995; Saxena & Stotzky, 2000; Brusetti et al., 2005). The alterations in the structure of root-associated and soil communities exposed to root exudates, detected by automated ribosomal spacer analysis (ARISA) (a fingerprinting method based on length variability of the intergenic region between rRNA genes) appeared to be caused by pleiotropic effects of the Bt gene, which is known for example, to result in a higher lignin content in maize (Saxena & Stotzky, 2001).

In a field test with a glyphosate herbicide-resistant canola variety, Dunfield & Germida (2001), and Siciliano & Germida (1999) found that the bacterial communities in the rhizosphere and in the inner root were significantly different than those in unmodified plants. These changes appeared to be temporary and restricted in terms of plant varieties (Dunfield & Germida, 2001, 2003). Also, rhizobacterial community patterns, and more specifically *Pseudomonas* populations of glufosinate-resistant canola, were slightly altered as observed by DGGE (Gyamfi et al., 2002). Although minor and restricted in time, these alterations were confirmed by reverse transcription DGGE, dot-blot hybridization and enzymatic activity analysis (Sessitsch et al., 2004). These results suggest that exudates of the transgenic plants were altered, and that they were responsible for the observed effects. In contrast, no such changes in community structure were observed by Schmalenberger & Tesbe (2003) using single-strand conformation polymorphism (SSCP) to analyze the rhizosphere of glufosinate-resistant maize.

Very few of the transgenic plants created to express other functions have been evaluated for their impact on rhizosphere communities. In those that were tested, such as alfalfa producing an alpha-amylase from *Bacillus licheniformis* or a Mn-dependent lignin peroxidase from *Phanerochaete chrysosporium* (Austin et al., 1995), slight or no effects of the transgene were detected. In some cases, the presence of a transgene resulted in selection for functionally equivalent but structurally different populations (Austin et al., 1995). Even the introduction of the T4 lysozyme into potatoes did not lead to significant differences between transgenic and control plant lines (Lottmann et al., 1999; 2000; Heuer et al., 2002), although the engineered plants were able to reduce plant pathogens such as *Pectobacterium atrosepticum* (During et al., 1993). Further manipulation of the rhizosphere was assessed when a *Pseudomonas putida* lysozyme-resistant fungal antagonist was introduced. While it colonized the transgenic plants better than the parental strain, a lysozyme-sensitive *Serratia grimesii* fungal antagonist was not affected by the transgene (Lottmann et al., 2000), suggesting that these strains do not interact. This sole example of the inoculation of a GM plant with a ‘cognate’ PGPR may suggest that manipulations have only limited effects on the rhizosphere.

In summary, the effect of transgenes appeared to be cultivar-dependent and line-dependent. Gene-dependent effects were less important (Donegan et al., 1995; Di Giovanni et al., 1999; Siciliano & Germida, 1999; Dunfield & Germida, 2001; Heuer et al., 2002). Moreover, these effects were almost always minor in comparison with environmental factors (Lukow et al., 2000; Heuer et al., 2002; Dunfield & Germida, 2004), suggesting that some unknown plant characteristics that may have been altered by the insertion of a transgene are responsible for the observed changes in microbial communities (Sessitsch et al., 2004). In any case, the ecological relevance of modifications in ‘transgenic rhizospheres’ has still to be evaluated. For this, predictions have to be made, and experimental models designed to confirm them.

**Concluding remarks**

Understanding ecological interactions is crucial for the proper use of inoculants, more so in the context of gene-modified organisms. This knowledge will be a guarantor of environmental and economic sustainability in which PGPR will replace polluting chemicals, and provide reproducible effects for the farmer year after year. These claims have been made for more than a generation. Fortunately, the use of PGPR on an unprecedented scale as is happening today is a comforting indication that useful PGPR technologies have finally emerged. To further strengthen this good harbinger, the public should be alerted of potential problems, so that the pros and the cons of new technologies can be weighted in a rational manner.

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References


Effect of inoculation with PGPR


