

# Free-living bacterial inocula for enhancing crop productivity

Joseph W. Kloepper, Ran Lifshitz and Robert M. Zablutowicz

Early work on free-living bacteria in soil indicated that certain strains, when applied to seeds or roots, may benefit crops by stimulating plant growth or by reducing the damage from soil-borne plant pathogens. Free-living bacteria may also influence the symbiosis between microorganisms and plants and thereby stimulate plant growth indirectly. This represents another potential commercial application for bacterial inocula within the next decade. However, fundamental work is still required to make bacterial inocula consistently effective.

Numerous microorganisms exert beneficial effects on plant development when applied to crop seed or incorporated into soil. This review focuses on one diverse group – the free-living bacteria. Symbiotic bacteria, such as *Rhizobium* spp. and beneficial fungi are discussed inasmuch as they interact with introduced free-living bacteria. We include within the broad coverage of 'free-living' the associative bacteria, such as *Azospirillum* spp., which may penetrate some root cells.

## Proposed mechanisms

Plant roots continuously exude nutrients which serve as food for soil microorganisms. In the early 1900s, the term 'rhizosphere' was coined for the zone within the root's influence. Rhizosphere microbiology soon became a popular research area with the realization that there are bacterial genera, such as *Pseudomonas*, that preferentially inhabit the rhizosphere. Some of the early studies documented beneficial effects on plant growth from mixtures of rhizosphere microorganisms<sup>1-3</sup>.

The descriptive studies on rhizosphere bacteria led to the identifica-

tion of specific bacterial physiological processes, such as nitrogen-fixation, that would be expected to benefit plant roots. It was hypothesized, therefore, that these processes were the reasons why inoculated plants had increased growth. *Azotobacter chroococcum* and various species of *Bacillus fix* atmospheric nitrogen<sup>4</sup> and in the mid 1970s, nitrogen-fixing strains of *Azospirillum*, which are naturally associated with roots of grasses, were evaluated extensively as inocula<sup>5-8</sup>. The resulting con-

sensus was that although many of these nitrogen-fixing bacteria can promote plant growth significantly, the primary mechanism is not nitrogen fixation<sup>4,6</sup>.

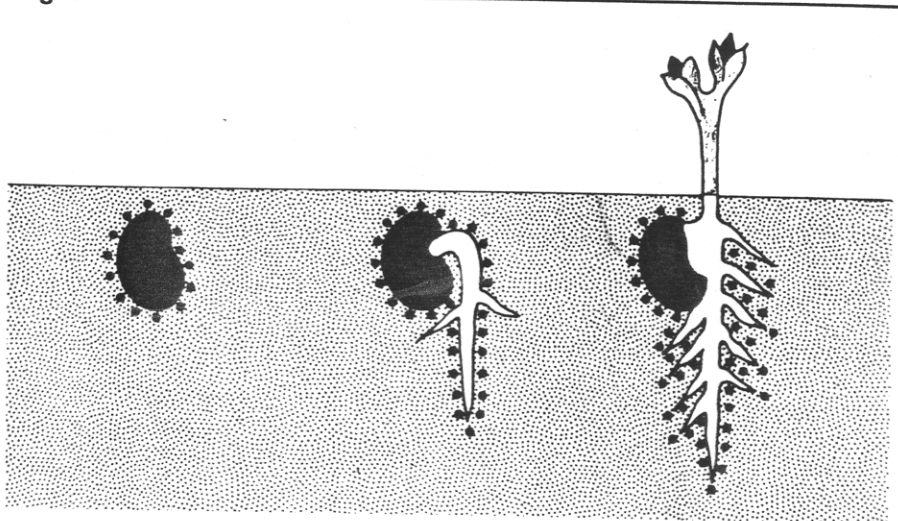
Another mechanism suggested for plant growth promotion by beneficial bacteria is mineralization of organic phosphorous compounds or solubilization of inorganic phosphorous compounds. These activities can be detected *in vitro* with numerous rhizosphere bacteria, and may increase phosphate availability to the plant<sup>9</sup>. Several phosphate-solubilizing or -mineralizing strains do promote plant growth although it is uncertain that this is because they make phosphate more available<sup>4,9</sup>.

Plant growth regulators, including gibberellins, cytokinins and indolyl-3-acetic acid (IAA), are produced *in vitro* by many soil bacteria. This, too, might constitute a mechanism for growth promotion by bacterial inocula<sup>4</sup>. While interesting indirect evidence suggests a role for plant growth regulators, we know of no evidence that shows a causal role.

## Rhizobacteria

A major barrier to the successful agronomic use of bacterial inocula is the need to establish high population densities of the introduced bacterium

Fig. 1



Root colonization. Bacterial inocula on seeds planted into field soils multiply in the seed zone in response to seed exudation before germination. Root-colonizers transfer from the seed zone to the developing root where they multiply and persist through the growing season.

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in the root environment, an environment characterized by intense microbial competition for nutrients<sup>10</sup>. The discovery and ecological characterization of 'rhizobacteria'<sup>11,12</sup> seemed to have overcome this barrier. Rhizobacteria are those members of the total rhizosphere bacteria that are able to colonize roots. Root colonization is defined as the bacterial capacity to multiply and keep pace with the growing root in field soil. From the practical point of view, it is important that colonization can follow as a result of inoculation (Fig. 1). Methods for monitoring bacterial root colonization, involving antibiotic-resistant strains<sup>12</sup>, have allowed detailed ecological analyses of bacterial root colonization under field conditions.

### Rhizobacteria as microbial inocula

The impact of rhizobacteria on plant growth and health may be neutral, deleterious or beneficial<sup>11</sup>. The term 'PGPR' – plant growth-promoting rhizobacteria – was coined in 1978<sup>13</sup> for beneficial rhizobacteria. Most PGPR are members of the fluorescent pseudomonads, but other types, including a non-fluorescent pseudomonad, an *Arthrobacter*-like strain and two *Serratia liquefaciens* strains have been described<sup>14</sup>. Their continual association with the crop root makes PGPR excellent candidates for delivering beneficial effects to the root zone.

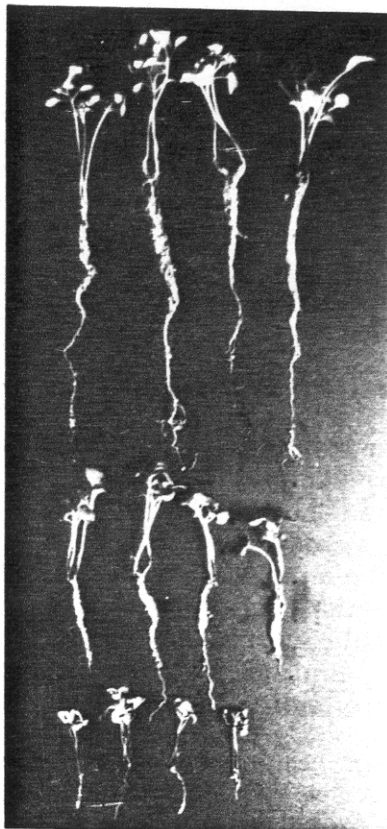
The beneficial effects of PGPR on crops fall into one of two categories – growth promotion or plant disease suppression.

#### Growth promotion

Growth promotion is evidenced by increases in seedling emergence, vigour, seedling weight, root system development and yield<sup>15</sup>. The mechanism first proposed for induction by PGPR of plant growth increases was the production of siderophores<sup>16</sup>, low-molecular-weight iron (III)-transport agents. Siderophores produced by various fluorescent pseudomonad PGPR reduce the availability of iron for deleterious rhizobacteria or fungi<sup>17,18</sup>, some of which inhibit plant growth under normal field conditions<sup>18</sup>.

The production by PGPR of toxic

Fig. 2



Biological control activity by a plant growth-promoting rhizobacteria (PGPR) strain. Canola (rape seed) was inoculated with a fungal pathogen, *Rhizoctonia solani*. The bottom row had no other treatment; the centre row was treated with a fungicide (PCNB); the top row received seed treatment with a pseudomonad PGPR strain.

compounds, such as antibiotics and HCN, which are active against deleterious rhizobacteria, has also been proposed as a mechanism for plant growth promotion<sup>10</sup>.

Not all strains of PGPR produce siderophores, antibiotics or HCN; therefore there must be other mechanisms. Indeed, we have described a strain of *Pseudomonas putida*<sup>19</sup> that promotes plant growth under gnotobiotic conditions (i.e. independent of deleterious rhizobacteria).

#### Biological control

Some PGPR strains have shown promise as biological control agents by reducing crop damage caused by major plant pathogens<sup>10,18</sup>. Most biological control via rhizobacteria has been directed towards soil-borne pathogenic fungi. One commercial product, containing a pseudomonad for control of damping-off disease on

cotton, was released in 1988<sup>10</sup>. (See Ref. 10 for a review of rhizobacteria as biological control agents.) An example of biological control activity of a PGPR strain is shown in Fig. 2. Not all bacteria that act as biological control agents are rhizobacteria. Many bacteria may persist in the root zone after inoculation without necessarily colonizing developing roots. Some of these have biological control potential<sup>10</sup>.

### Yield effects in field trials

The results of field trials with bacterial inocula reported before 1974 have been reviewed<sup>4</sup>. During the 1950s, there were extensive field trials in the Soviet Union with several bacterial types, primarily *Azotobacter chroococcum* and *Bacillus megaterium*. By 1958, about 10<sup>7</sup> ha had been treated with bacterial inocula; increases in yield of 10–20% were reported in 50–70% of the trials. Since the early 1960s, scientists elsewhere have conducted comprehensive and statistically designed experiments with different crops, the inocula being mainly species of *Azotobacter* and *Bacillus*. These experiments essentially confirmed the Soviet reports; bacterial inocula increased yields of many crops in many trials. However, the effects were inconsistent.

One representative study<sup>20</sup> evaluated the effect of bacterial inoculants on wheat. *Azotobacter* inoculation resulted in changes of average yields ranging from a 10% decrease to a 30% increase over controls, while results from *Bacillus* inoculation ranged from a 6% decrease to a 43% increase. There was a higher tendency for positive than negative effects, with 29 out of 71 trials giving over 5% higher yield, while only four trials gave over 5% lower yield than controls.

Since 1974, field research work with bacterial inocula has focused more on biological control of plant diseases than on growth promotion. Such studies use bacteria, including fluorescent pseudomonads and *Bacillus subtilis*, that are antagonistic to soil-borne plant pathogens<sup>10</sup>. There were also extensive inoculation trials after 1974 using free-living nitrogen-fixing bacteria, but interest

**Table 1**  
**Ranges of reported crop yield increases with bacterial inocula in field reports involving multiple trials after 1974**

Crop	Range of % yield difference from control*		Ref.
	lowest	highest	
<b>Azospirillum spp.</b>			
Maize	6.7	75.1	a
Millet	-12.1	31.7	b
Mustard	16	128	c
Rice	4.9	15.5	d
Sorghum	12	18.5	e
Sorghum (and millet)	-4	24	f
Sorghum	20.5	30.5	a
Wheat	-15.8	31	g
Wheat	-8.5	25.8	h
Wheat	-2.5	19.6	i
Wheat	-9.6	14.8	j
<b>Bacillus spp.</b>			
Peanut	-6.1	37.4	k
Potato	-16	12	l
Sorghum	15.3	33	m
Wheat	0	114	n
<b>Pseudomonas putida, P. fluorescens</b>			
Canola	0	57	
Potato	-14	33	n
Potato	-10	37	p
Potato	0	17	q
Potato	2.9	6.7	r
Potato	-9	20	s
Potato	-17	11.7	t
Radishes	0	100	
Rice	3	160	v
Sugar beet	-11	32	w
Wheat	1.9	26.3	x

\*The values are based on the data presented in each report. No distinctions were made between strains, cultivars of a given crop, soil types, fertilizer regimes, environmental variables or delivery systems.

<sup>a</sup>Kapulnik, Y. et al. (1981) *Exp. Agric.* 17, 179-187; <sup>b</sup>Bouton, J. H. et al. (1979) *Crop Sci.* 19, 12-16; <sup>c</sup>Saha, K. C. et al. (1985) *Plant Soil* 87, 273-280; <sup>d</sup>Rao, V. R. et al. (1983) *J. Agric. Sci.* 100, 689-691; <sup>e</sup>Sarig, S. et al. (1988) *J. Agric. Sci.* 110, 271-277; <sup>f</sup>Smith, R. L. et al. (1984) *Appl. Environ. Microbiol.* 47, 1331-1336; <sup>g</sup>Baldani, V. L. D. et al. (1987) *Biol. Fert. Soils* 4, 37-40; <sup>h</sup>Kapulnik, Y. et al. (1987) *Biol. Fert. Soils* 4, 27-35; <sup>i</sup>Kapulnik, Y. et al. (1983) *Can. J. Microbiol.* 29, 895-899; <sup>j</sup>Reynders, L. and Vlassak, K. (1982) *Plant Soil* 66, 217-223; <sup>k</sup>Turner, J. T. PhD thesis, Auburn University (1987); <sup>l</sup>Burr, T. J. et al. (1978) *Phytopathology* 68, 1377-1383; <sup>m</sup>Broadbent, P. et al. (1977) *Phytopathology* 67, 1027-1034; <sup>n</sup>Capper, A. L. and Campbell, R. (1986) *J. Appl. Bacteriol.* 60, 155-160; <sup>o</sup>Kloepper, J. W. et al. (1988) *Plant Dis.* 72, 42-46; <sup>p</sup>Howie, W. J. and Echandi, E. (1983) *Soil Biol. Biochem.* 15, 127-132; <sup>q</sup>Kloepper, J. W. et al. (1980) *Phytopathology* 70, 1078-1082; <sup>r</sup>Leben, S. D. et al. (1987) *Phytopathology* 77, 1592-1595; <sup>s</sup>Geels, F. P. et al. (1986) *Neth. J. Plant Pathol.* 92, 257-272; <sup>t</sup>Xu, G. W. and Gross, D. C. (1986) *Phytopathology* 76, 423-430; <sup>u</sup>Kloepper, J. W. and Schroth, M. N. (1978) in *Proceedings of the 4th International Conference on Plant Pathogenic Bacteria*, pp. 879-882; <sup>v</sup>Sakthivel, N. and Ganamanickam, S. S. (1987) *Appl. Environ. Microbiol.* 53, 2056-2059; <sup>w</sup>Suslow, T. V. and Schroth, M. N. (1982) *Phytopathology* 72, 199-206; <sup>x</sup>Weller, D. M. and Cook, R. J. (1986) *Can. J. Plant Pathol.* 8, 328-334.

### Statistical analysis of field trial data

The potential of a given strain to promote plant growth may be determined in a single, well designed field experiment, using statistical analysis of variance (ANOVA). If the mean yield with the treatment is greater than the control yield in a replicated field trial, the data are evaluated with the appropriate ANOVA (or other statistical test). If the mean difference of the treatment is found to be significant at a level of  $p = 0.05$  or lower, it is readily accepted that the effect is real. However, each evaluation of efficacy applies only to the conditions of that field trial and not to others in different years and/or different locations.

Hence, the potential of a bacterial inoculum may be determined in a single experiment, but the consistency of performance can only be determined in multiple trials (Fig. 3). Consistency has rarely been addressed experimentally by statistical analysis across multiple trials. Our view is that a proper evaluation of consistency is necessary to assess the real potential of microbial inocula.

Evaluation of consistency is necessary not only to characterize the quality of inocula, but also to identify the causes of inconsistency. Frequently, failures in inoculation trials were 'rationalized' by logical explanations given after the event. Subsequent trials did not always confirm the source of inconsistency. It is, therefore, likely that other, unknown environmental and/or plant genotype variables affect the performance of bacterial inocula. Such 'inconsistency variables' may be identified when more trials are repeated and a universal data analysis of consistency is accepted. Knowing more about inconsistency could provide a basis for guiding product development.

### Effects of inocula on rootzone microorganisms

Some free-living bacterial inocula can positively interact with various plant-symbiotic microorganisms, such as *Rhizobium*, *Bradyrhizobium*, *Frankia* and mycorrhizal fungi (Table 2). The resultant microbial synergism can enhance plant growth.

has shifted from *Azotobacter* towards *Azospirillum* which has a closer association with roots<sup>5</sup>. Numerous studies world-wide reported statistically significant yield increases (Table 1).

Today, there is no doubt that bacterial inocula can increase the yield of various crops significantly, but performance has generally been inconsistent. This is not surprising, considering the complexity of the

system. Bacterial effects on plant growth result largely from multiple interactions between the introduced bacteria, the associated crops and the soil microflora. Each of these interactions is determined by multiple environmental variables such as the soil type, nutrition, moisture and temperature. Inconsistency of performance is still a major hurdle for the commercial development of bacterial inocula.

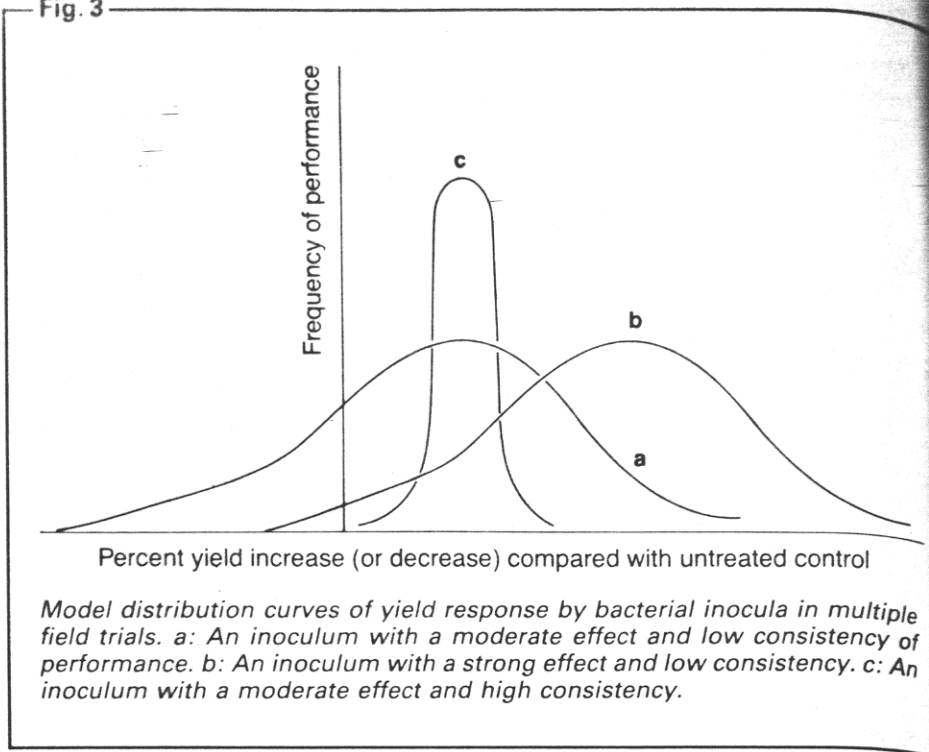
In the 1940s certain rhizosphere bacteria, termed 'activating bacteria', were reported to enhance the rate and extent of nodulation and nitrogen fixation by *Rhizobium trifolii*, and the subsequent yield of inoculated clover<sup>21</sup>. Similarly, some rhizosphere bacteria increased nodulation by a 'weakly virulent' *Rhizobium* strain compared with nodulation in an otherwise sterile soil system<sup>22</sup>. Other work<sup>23</sup> indicated that rhizosphere bacteria enhanced nodulation caused by *R. meliloti* and *R. trifolii* more than they enhanced nodulation by *R. lupini*.

In both actinorrhizal- and endomycorrhizal-plant symbiosis, the symbionts (*Frankia* spp. and various genera of fungi) do not readily infect the plant under sterile conditions. Co-inoculation with *Pseudomonas cepacia* and a *Frankia* sp., however, improved both the consistency and frequency of nodulation of aseptically cultured alder<sup>24</sup>. Aseptically grown clover was considerably less infected when inoculated only with spores of an *Endogone* sp. than were plants previously contaminated with *Pseudomonas*<sup>25</sup>. Thus 'activating bacteria' may have roles beyond the stimulation of the rhizobial symbiosis.

The competition of *Rhizobium* spp. inocula with native rhizosphere microorganisms (including indigenous rhizobia) may limit the introduction of new strains. One way of reducing competition might be to make use of antibiotic sensitivity in soil organisms. Li and Alexander<sup>26</sup> inoculated three antibiotic-producing bacteria and antibiotic-resistant rhizobia on alfalfa and soybeans. Rhizobial colonization, nodulation and plant biomass were significantly enhanced when antibiotic-resistant rhizobia were co-inoculated with a pseudomonad or *Bacillus* sp., while a *Streptomyces* sp. elicited higher nodulation only with the addition of chitin. In another study<sup>27</sup>, however, both an antibiotic-producing strain (*Pseudomonas putida* M17) and a mutant (M174) that does not produce antibiotic significantly enhanced the nodulation of beans in greenhouse and field trials.

Many beneficial bacteria produce a wide range of phytohormones

Fig. 3



(auxins, gibberellins and cytokinins) and enzymes, such as pectinase, that are involved in the infection process of plant-microbe symbiotic systems. For example, *Azospirillum* produces both IAA and pectinase and can stimulate or decrease nodulation by *Rhizobium*<sup>28,29</sup>; however, a causal relationship has not been confirmed. Stimulation of *Rhizobium* nodulation by *Azospirillum* is clearly associated with alterations in root morphology, which are caused by the *Azospirillum*<sup>28,29</sup>.

There is renewed interest in the interactions of the root-colonizing rhizobacteria with symbiotic microorganisms. Their close association with roots throughout the life-cycle of the plant and their capacity to stimulate plant growth, makes PGPR good candidates for root-zone delivery of compounds that could enhance the activity of various plant symbionts. *Bacillus subtilis* strain A-13 is a PGPR which is moderately competitive on the root (J. T. Turner, PhD thesis, Auburn University, 1987). On peanut, A-13 stimulates root branching and, in the presence of *Rhizobium*, results in increased nodule numbers and enhanced nitrogen levels in the plant. In another study<sup>30</sup>, 17 rhizobacteria strains (*Pseudomonas fluorescens* and *P. putida*) produced antibiotics effective *in vitro* against the soybean rhizobium, *Bradyrhizobium japonicum*. In co-inoculation soil studies, all 17 strains colonized roots aggres-

sively (log 3.9–5.7 c.f.u.g<sup>-1</sup> root); but none significantly reduced soybean nodulation, and six significantly increased nodule mass with *B. japonicum* strain 110. Thus combined inocula consisting of rhizobia and rhizobacteria have potential for enhancing legume development.

Inoculum combinations including mycorrhizal fungi may also be useful. Mycorrhizae have been associated with both plant growth promotion and biological control of soil-borne pathogens<sup>31</sup>. There are preliminary reports of synergistic reactions under greenhouse conditions<sup>32</sup>. Linderman<sup>31</sup> suggests that inconsistencies in PGPR activity may result in part from mycorrhizal interactions.

A multicomponent inoculum for legumes, consisting of *Rhizobium*, mycorrhizae and a free-living bacterium, has also been investigated<sup>33,34</sup>. Plant biomass was increased by each component individually, and the greatest response occurred when all three components were present. The free-living bacteria and the mycorrhizae each increased nodulation and nutrient uptake but when they were used in combination a significant synergy resulted<sup>34</sup>.

#### Caveats

Conceptual mechanisms of action of bacterial inocula have become accepted as facts without necessarily being supported by critical data. A logical hypothesis and indirect, circumstantial supporting evidence

Table 2

## Possible mechanisms for enhancement of plant-microbial symbiotic relationships by free-living bacteria

Mechanism	Example
Antibiosis	Enhancement of the symbiont's rhizosphere competitive ability by antagonizing other rhizosphere microflora
Plant growth regulators, enzymes	Enhancement of plant susceptibility to the symbiont or increasing the symbiont infection
Biological control	Controlling plant diseases that affect root density or vascular function and which can limit sites or substrate for symbiont infection and maintenance
Inhibitors	Production of bacterial compounds that interfere with plant regulation of the symbiosis resulting in increased infectivity and symbiotic activity
Nutrition	Enhancing the availability of nutrients that may improve the efficiency of the symbiosis
Detoxification	Metabolism of compounds present in the soil or rhizosphere that may interfere with the symbiosis

The examples are theoretical models by which a free-living bacterial inoculum could cause increased activity of the symbiotic microorganism. Symbiotic microorganisms may include *Rhizobium*, *Bradyrhizobium*, *Frankia* or mycorrhizal fungi.

ships<sup>10,17</sup>. However, authors should clearly distinguish between facts and speculations. Accordingly, new *in-vitro* strain selections, or genetic engineering programs based on improving characteristics whose significance has not been clearly shown, may not be meaningful.

### Product development challenges

The number of corporate research and development groups with an agricultural microbiology focus is rapidly increasing. These groups recognize that bacterial inocula will be developed in two 'generations' – the first consisting of naturally occurring bacteria, and the second consisting of genetically engineered bacteria.

Several challenges must be resolved before the full product potential of bacterial inocula is realized. The challenge of developing consistent benefits of inocula has already been discussed. Progress will continue to be made as modes of action are elucidated on a case-by-case basis. Another principle challenge is delivering the product. Appropriate formulations must not only maintain viability of the bacteria but also sustain growth promotion or biological control activities of the inoculum<sup>35</sup>. The formulation must then be developed into a delivery system that allows application by farmers or seed companies within existing equipment and practices<sup>35</sup>.

Fermentation systems for producing the inocula must also be optimized and the quality of their output controlled with respect to inoculum density and biological activity. Thus rapid assays for biological activity (growth promotion or biological control) are required for use during product development and production. Regional field trials must be conducted with the product prototype, and the environmental limits on biological activity must be determined. The survival and dispersal of bacteria in the environment must be monitored.

Advances in our understanding of modes of action are likely to come from the public sector, while those concerning formulation and delivery system advances will probably come from the private sector. Collaboration

do not constitute a basis for subsequent R&D efforts. Some of the conceptual mechanisms are attractive and may lead to experiments that are (unconsciously) designed to support, rather than challenge, the concept. This can only impede progress towards applications. The concepts may have merit but they have not been tested as critically as they need to be.

One such concept is that biological control of plant diseases by rhizobacteria results from the same antagonism in the root zone as that observed *in vitro*. For plant pathologists it is quite logical to test bacteria that are antagonists of plant-pathogenic fungi *in vitro* as potential biological control agents. There have been numerous attempts to exploit such bacteria<sup>10</sup>. Strains of *Bacillus subtilis*, fluorescent *Pseudomonas* spp. and other species that are fungal antagonists *in vitro* have been evaluated in field trials and shown to be effective. But the general assumption that the principal mechanism of disease control is the same as for antagonism observed *in vitro* frequently lacks convincing experimental support. (A few exceptions are pointed out in the review by Weller<sup>10</sup>.) *In-vitro* antagonism is not necessarily linked to biological control effect in field soil because:

- There are significant differences in the behaviour of microorganisms *in vitro* and in the soil.

- Appropriate controls (organisms that were not antagonists *in vitro*) have not been examined. Thus we know little about the biological control potential of *in-vitro* non-antagonists.

- Some of the best-known biological control agents [for example, *Bacillus subtilis* A13 (J. T. Turner, PhD thesis, Auburn University, 1987) and *Pseudomonas putida*<sup>19</sup>] promote growth in the absence of pathogens. The observed reduction in disease severity may then be merely a secondary effect resulting from growth promotion.

Other commonly accepted concepts which, we believe, have not been critically assessed derive from ecological considerations: 'root colonization is required to observe consistent beneficial effects of free-living bacterial inocula'; 'inconsistent field performance by PGPR and biological control agents can be largely explained by inconsistent and inferior root colonization'; 'according to the principles of microbial ecology, the best place to search for a biological control agent is in the same ecological niche as the intended target pathogen'.

Causal relationships between *in-vitro* physiological processes of bacteria and growth promotion in the field must be shown as directly as possible. Tn5 mutagenesis techniques provide a powerful tool for helping to establish causal relation-

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