Nitrogen Assimilation by Plants

Physiological, Biochemical and Molecular Aspects

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Plant Growth Enhancement by Rhizobacteria

J.C. Cleyet-Marel, M. Larcher, H. Bertrand, S. Rapior and X. Pinochet

The action of plant growth-promoting rhizobacteria, known as PGPR, falls into two broad categories: direct plant growth stimulation and biological control. Several reviews summarise work on PGPR as biological control agents (Kloepper, 1993; Glick, 1995). The focus here is on the direct plant growth promotion mechanisms by PGPR. Inoculation of plants with PGPR at an early stage of development results in positive impact on biomass production through direct effects on root growth, morphology and physiology. These results are described for both laboratory and field experiments. Changes in root morphology and physiology have been extensively investigated in laboratory conditions; promotion in development of the root system is much more difficult to assess in the field. Measurements of the growth promotion effect are carried out on agronomic parameters, mainly plant growth, grain yield and nitrogen content. The direct growth promotion effect may be attributed to many factors which act synergistically. The main factors proposed in the literature are: (i) production of phytohormones or other compounds by the bacteria which can act directly on plant growth; (ii) mineral enhancement uptake and (iii) transfer of nitrogen to the plant. Rhizosphere bacterial populations and their physiological activities therefore contribute considerably to primary production in terrestrial ecosystems and need more consideration in the perspective of sustainable agriculture.

DIVERSITY IN PLANT GROWTH-PROMOTING RHIZOBACTERIA

Among the non-symbiotic rhizobacteria, those that are free-living in the soil but often found near, on, or even within the roots of plants are drawing more and more attention. Since their rediscovery in the 1970s, bacteria of the genus *Azospirillum* have been extensively studied both in the laboratory

and in the field. Nevertheless, a number of other bacteria may be considered to be PGPR, including *Azotobacter* species, pseudomonads, *Acetobacter* species, *Burkholoderia* species, rhizobia and bacilli. The list of PGPR other than the aforementioned increases annually as more research groups engage in screening rhizosphere bacteria for plant growth promotion. Hence the potential for promoting plant growth is broadly distributed within the constituent groups of the rhizosphere bacterial community. In addition, among the bacteria identified on the root surface (rhizoplane) and in the rhizosphere, a significant number of bacterial species are detected in the root interior (endophytic bacteria). The relationship between the bacterial communities associated with the root surface and the root interior has not been fully understood to date; the endophytic bacterial species may be either a subset of those found on the root surface or a distinct group yet to be identified.

DIRECT GROWTH PROMOTION MECHANISMS

The mechanisms by which bacterial cells affect plant cells are still misunderstood. Several effects on root morphology and functions have been suggested, e.g. biological N_2 fixation at different magnitudes of importance, production of growth substances by the bacteria that develop the reproductive parts, stimulation of plant growth, involvement in promotion of root branching and root-hair development, thereby increasing root exudation.

But none of the aforesaid mechanisms is entirely accepted as a major contribution of bacteria. Currently accumulating data indicate that several mechanisms, each small in magnitude, may contribute simultaneously to the overall effect observed in plants (Fig. 11.1). Plant response is obviously a very complex phenomenon and each study brings a bit of understanding. However, a few major phenomena are clearly linked with plant response.

Diazotrophic Bacteria

Reports of very high N_2 fixation in the tropical grass-*Azospirillum* system in Brazil in 1974 stimulated renewed interest in associative N_2 fixation. These results came from several workers who determined whether associative N_2 fixation would benefit plant productivity and reduce nitrogenous fertiliser requirements. Subsequent experiments throughout the world using total nitrogen concentration, acetylene reduction activity (ARA), or ^{15}N isotope dilution to measure N_2 fixation, yielded various conflicting results about the importance of these bacteria as crop inoculants. In a field experiment on heavy clay soil where ten winter wheat cultivars were tested for yield response to inoculation with *A. brasilense*, it was reported that 68 to 87 kg of mineral nitrogen per hectare could be saved by inoculation (Reynders and Vlassak, 1982). In field experiments with *A. brasilense* inoculation of *Sorghum* and *Pennisetum* species, inoculation response appeared to be affected by the rate of nitrogenous fertiliser applied; responses were noted only in the

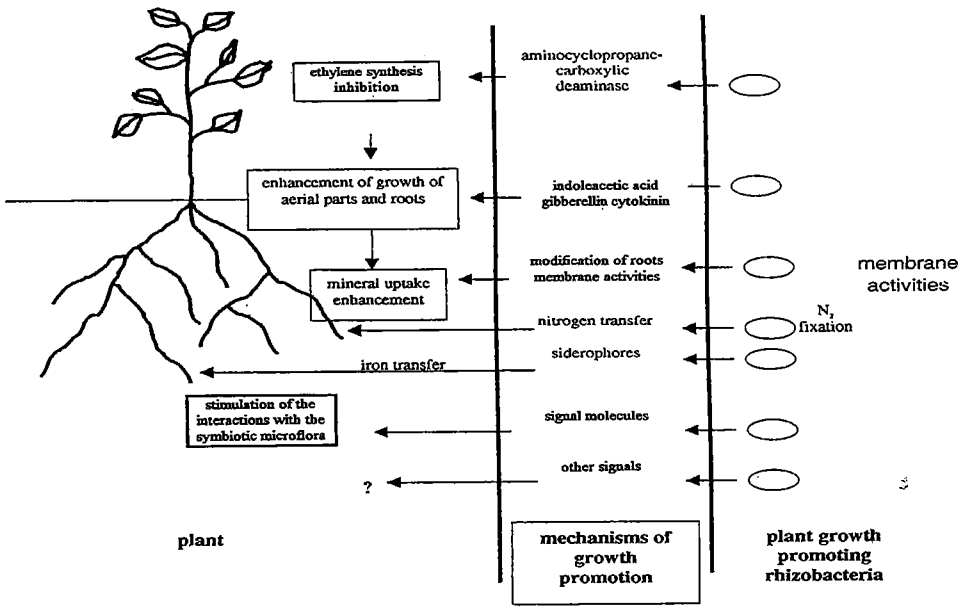

Fig. 11.1 Schematic representation of the possible mechanisms of plant growth promotion by rhizobacteria.

intermediate rates of nitrogenous fertiliser. The ARA in these investigations was low, site dependent and correlated with soil moisture. Although acetylene reduction activity is not the most appropriate approach for evaluating N_2 fixation, the data reported from the aforesaid study suggest that N_2 fixation is not important for plant growth response. The ^{15}N isotope dilution technique gave evidence that plants inoculated with *Azospirillum* obtain approximately 5% of their nitrogen from the fixation process. Using a wild-type and a site-directed mutant strain (Nif) of *A. brasilense* totally deficient in N_2-fixation capability, Bashan *et al.* (1989) demonstrated that the contribution of *Azospirillum* Cd to growth of tomato seedlings was not through the N_2-fixation process. Many other studies with *A. brasilense* suggest that N_2 fixation is not the major mechanism whereby growth response to inoculation is achieved (Okon and Kapulnik, 1986; Tien *et al.*, 1979).

Enhancement of Cell Division and Root Elongation Zone

A significant enhancement of cell division in root tips 24 hours after inoculation and an increase in size of the elongation zone 48 hours after

inoculation was demonstrated in plants inoculated with *Azospirillum brasilense* strain Cd. Both effects occurred immediately after germination; their magnitude was lower when measured at later stages of development. These findings corroborate a previously suggested hypothesis that the marked effect of *Azospirillum* on plant development occurs in the initial stages of seed germination. In a model system with *Arabidopsis thaliana* and *A. brasilense*, it was shown that root hair length of inoculated and non inoculated seedlings measured at physiologically identical stages of root growth was more than twice the length of hairs of the non-inoculated control (Dubrovsky *et al.*, 1994). Calculations based on data from the literature on shoot and root mass of grasses (79 plant/bacteria associations analysed) revealed that inoculation with *Azospirillum* spp. increased the shoot-to-root (S/R) ratio in about half of the reported cases and decreased the S/R ratio in the other half (Bashan and Dubrowsky, 1996). Nitrate uptake from the nutrient solution measured during plant ontogeny (about every 10 days) increased significantly when plants possessed three leaves and until the flowering stage. The increases observed in this system seem to be due to a general increase in root area and not to a specific uptake rate; thus, the main benefit of inoculation appears to be based on an enhancement in root development, starting immediately after germination.

Mineral Uptake Enhancement

Several reports indicate that plant nutrient uptake can be stimulated by inoculation. A high increase of K^+ and $H_2PO_4^-$ uptake was also observed in 1-week-old hydroponically grown corn seedlings inoculated with *A. brasilense* 48 hours after inoculation (Lin *et al.*, 1983). Based on measurements of root segments of young corn seedlings, the enhanced ion uptake phenomenon was extended to K^+ and $H_2PO_4^-$. No gross changes in root morphology or root weight were observed but alteration of cell arrangement in the outer four to five layers of the cortical cells of corn roots from 3-day-old corn seedlings was found. Inoculation of seedlings of rice with nitrogen-fixing *Azospirillum lipoferum* induced an increase in uptake of NH_4^+ ion. The increase was significant during the first week after inoculation but decreased both in control and inoculated plants 21 and 28 days after inoculation. The inoculated plants showed enhanced PO_4^- ion uptake over control from 7 days after inoculation to the end of the experiment (Murty and Ladha, 1988). Testing *Azospirillum* spp. inoculation on root development and NO_3^- uptake in wheat in the hydroponic system, Kapulnik *et al.* (1985) obtained a significant enhancement of root elongation for inoculated plants compared with non-inoculated controls.

In an exhaustive experiment, Bashan *et al.* (1990) examined whether a general enhancement in uptake of minerals other than nitrogenous compounds was a common mechanism induced in plants by PGPR; the

authors observed no consistent pattern of increased ion content of plant tissues in any bacterial strain-plant combination evaluated.

In a study involving oilseed rape (*Brassica napus*) and an *Achromobacter* strain, Bertrand *et al.* (2000) reported a 100% increment in NO_3^- depletion from the medium and NO_3^- uptake rates by the inoculated oilseed rape seedlings compared to non-inoculated seedlings (Fig. 11.2). Using ^{15}N labelling, it was found that inoculated plants accumulated ^{15}N in roots and shoots more than non-inoculated plants. Absorbed ^{15}N was rapidly transported and after two hours, at least twice the amount of ^{15}N was found in the shoots than in the roots. Considering that no modification of length and diameter of the seminal root nor increase in biomass of the root system was observed in response to inoculation, it is unlikely that the increase of NO_3^- uptake was due to root hair modifications. It was then hypothesised that a stimulation of the specific NO_3^- rate occurs. Using NO_3^- selective microelectrodes, it was shown that the *Achromobacter* isolate 3-17 triggered an enhancement in NO_3^- influx measured in the root portion between 3 and 10 cm away from the apex. These results demonstrate that the bacteria enhance NO_3^- uptake rate per se, independent of changes in root surface area. One possible explanation to account for this response pattern is that NO_3^- transport systems could have been up-regulated in the roots of oilseed rape plants inoculated with the *Achromobacter* strain. The uptake of NO_3^- is a net flux, the difference between an influx and an efflux-across the plasma membrane of epidermal and cortical cells of the roots. Although the efflux component was of the same order of magnitude as the influx, the NO_3^- efflux system was relatively stable as long as NO_3^- was present (Aslam *et al.*, 1996), and the variations of NO_3^- uptake were explained as due to regulation of influx (Hole *et al.*, 1990; Muller *et al.*, 1995).

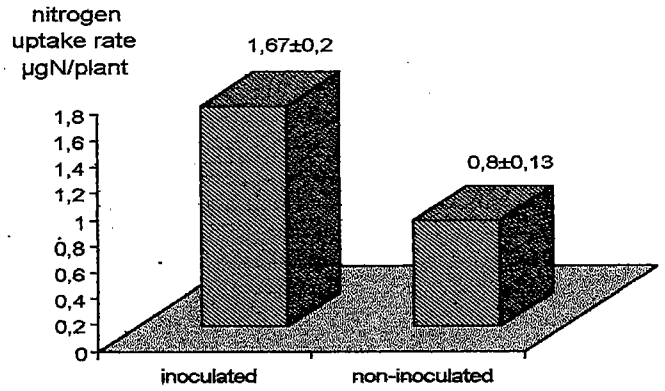

Fig. 11.2 Nitrogen uptake by oilseed seedlings inoculated or non-inoculated estimated by the disappearance of NO_3^- in the medium over a 2-hour period (μg N plant^{-1}).

Physiological studies showed that at least three different types of NO_3^- transporters, each referred to as a transport system, coexist in the plasma membrane of the root component. These include a low-affinity transport system (LATS), which makes a significant contribution to unregulated NO_3^- uptake at high external NO_3^- concentration; two high-affinity transport systems operate at low external NO_3^- concentration ($5 < K_m < 200$mM), one constitutive (CHATS), the other inducible by NO_3^- ions (IHATS). The NO_3^- uptake rate in non-induced oilseed rape plants (i.e., never supplied with NO_3^- before commencement of the experiment) increased threefold after inoculation with the 3-17 strain (Fig. 11.3). This suggests that the *Achromobacter* sp. 3-17 acts on the NO_3^- constitutive high-affinity transport system.

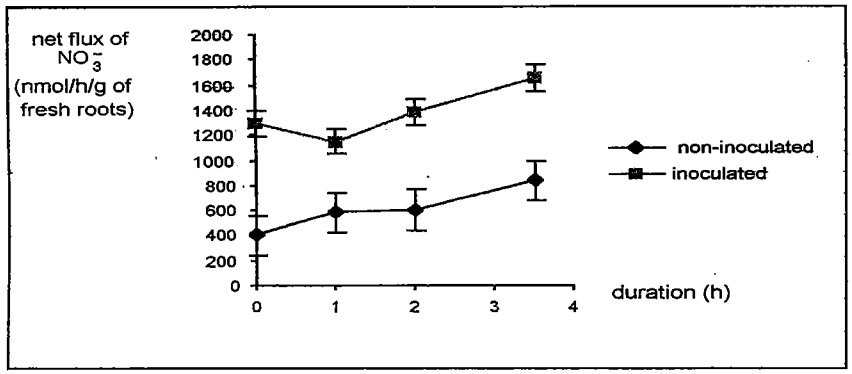

Fig. 11.3 Net flux variations of NO_3^- in oilseed seedlings inoculated or not inoculated with isolate 3-17, 24 h before the experiment. Plants were in contact with nitrate for the first time at T_0. Measurements were done at 5.5 cm away from the apex. Results are the average of 3 plants. Vertical bars represent standard error.

Increases in both K^+ net influx and H^+ net efflux were likewise observed in inoculated plants when these fluxes were measured at different places on the seminal roots using specific microelectrodes. An enhancement of proton efflux was also reported in wheat seedlings inoculated with *Azospirillum* (Bashan, 1990). Protons are extruded from the cells by a plasma membrane Mg-ATPase which functions as an electrogenic pump that is the master system for driving uptake of essential nutrients, providing the ion transporters with energy (Grignon, 1990). Depending on the overall balance of anion and cation net uptake rates, either a proton net efflux or net influx is measured. In any case, the fact that inoculation with *Achromobacter* sp. 3-17 led to increased net efflux of H^+ strongly suggests that the proton pump activity was enhanced in the roots of inoculated plants. If that could be

proven, this effect might well result in increase in rates of transport of every ion absorbed, including K^+ and NO_3^-.

Another possible explanation for the increase in NO_3^- uptake (and possibly in K^+ uptake) could be that the growth-promoting effect of the bacteria induced an increased nitrogen (and potassium) demand level in rapeseed oil plants. Such a change in nutritional demand of plants is indeed known to enhance NO_3^- uptake rate (Ismande and Touraine, 1994).

Plant Growth Hormones and Other Compounds

Among the possible mechanisms proposed to explain the beneficial effects of free-living bacteria on plant growth are phytohormone production and some specific enzymatic activities. The release of phytohormones has been proposed as responsible for proliferation of the host-plant root system. It has also been reported that *Azospirillum* releases the phytohormone indole-3-acetic acid (IAA) into the culture medium in the presence of tryptophan. It was suggested that only auxins are important in promoting plant growth on the premise that bacteria produce higher amounts of IAA-like compounds than gibberellin- and cytokinin-like substances. However, extremely low concentrations of gibberellin seem to be involved in root growth promotion and their significance should not be underestimated. The proposed implication of IAA came from results obtained by biological assays in which the effects could be mimicked by exogenous auxins. A comparison between the actions of rhizobacteria and pure IAA on root growth of plants demonstrates that the effects of the bacteria can be ascribed to the action of IAA. Like root elongation, the production of laterals in all root types is equally affected by IAA application and inoculation with IAA-producing rhizobacteria. Through the enhancing effect of IAA and IAA-producing bacteria on root branching, the total root length of the plants increases. Strains with a strong effect on root growth also enhance the accumulation of certain nutrients. Direct evidence that *A. lipoferum* inoculation affects the gibberellin status of corn seedling roots was given by Fulchieri *et al.* (1983). More direct evidence for the role of the bacterial IAA production has been reported. For instance, a mutant strain producing more IAA (thereby reducing the ability to promote root system development) than the wild-type strain has been reported (Barbieri and Galli, 1993). IAA biosynthesis in bacteria appears to be quite complex. Analyses of tryptophan (Trp), indole-3-acetamide (IAM) and indole-3-acetic acid (IAA) bioproduced by the mutant strain SpM7918 showed an indoleacetamide accumulation concomitant with reduced indoleacetic acid synthesis (Prinsen *et al.*, 1993). Using HPLC with on-line mass spectrometry and radioactive labelling, multiple IAA biosynthetic pathways were revealed in *Azospirillum*, such as the indoleacetamide pathway, a second tryptophan-dependent pathway and a tryptophan-independent pathway, the latter predominant when no tryptophan was supplied to the medium. Bothe *et al.* (1992) put forward an alternative explanation for root growth promotion by *Azospirillum*. The effects

of *Azospirillum* on wheat root morphology can be better correlated with those caused by nitrite. Both *Azospirillum* and nitrite have a slight positive effect on dry weight, cause a drastic increase in ratio of lateral roots but exert no significant effect on the formation of root hairs/lateral roots. According to Bothe *et al.* (1992), the bacterium produces nitrite in large quantities during nitrate respiration and this nitrite may interact in plant cells with ascorbic acid to form a phytohormonal active compound. *Pseudomonas putida* GR12-2, another PGPR bacterium, possesses 1-aminocyclopropane-1-carboxylic (ACC) deaminase activity. Three mutants obtained with nitrosoguanidine are devoid of the ACC deaminase activity and all of them impaired in the ability to stimulate root elongation of canola seedlings (Glick *et al.*, 1994). These authors suggest that the ability of *P. putida* to stimulate root elongation is due, at least in part, to ACC deaminase activity. The simplest model to explain this observation is that *P. putida* hydrolyses ACC, the precursor of ethylene, thereby lowering the level of ethylene in a developing plant.

Microbial Siderophore in Iron Acquisition by Plants

Siderophores are iron-chelating agents that can increase and regulate the availability of iron in the plant rhizosphere. Plants might have siderophore-iron transport systems or, under reduced soil conditions, might acquire inorganic iron buffered by dissociation in the plant rhizosphere. Siderophores are also microbiologically produced and involved in plant nutrition since they serve as iron sources for plants. Over 80 different siderophore-producing bacteria and fungi have been isolated but not all siderophores are used by plants; individual plant species and varieties differ in ability to utilise specific siderophore types. In soils, microbial siderophores have much higher affinity for iron than plant-produced organic acids or phytosiderophores. Results obtained by Crowley *et al.* (1987) confirm the existence of a siderophore-mediated iron transport system and suggest that siderophores produced by *Pseudomonas* sp. can supply iron to plants that have mechanisms for using these compounds under iron-limiting conditions.

POTENTIAL APPLICATIONS

The uses of rhizobacteria for enhancing crop productivity should be based on optimisation of their application and success in root colonisation to enable plant-rhizobacteria interaction. Root colonisation of the rhizobacteria and their survival in the soil are certainly the main limiting factors in the application of PGPR (Fig. 11.4).

Root Colonisation

Since rhizobacteria do not result in the formation of any readily detectable plant structure, the ability of an introduced rhizobacterium to colonise roots

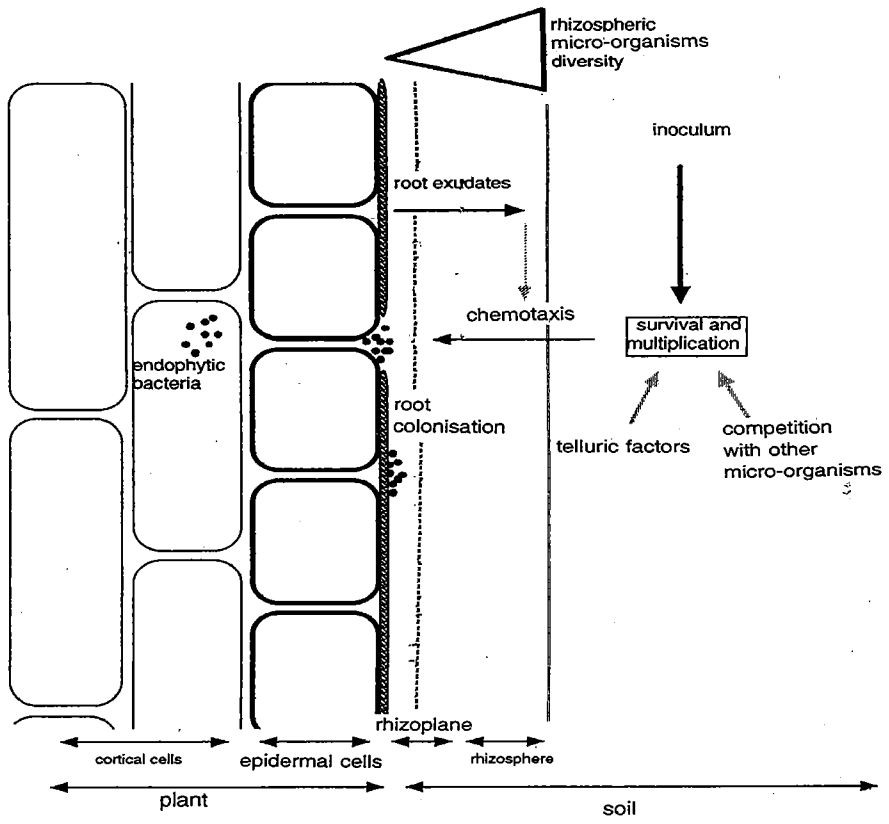

Fig. 11.4 Schematic representation of root colonisation by plant growth-promoting rhizobacteria.

must be evaluated. To demonstrate the establishment, multiplication and functioning of the introduced micro-organisms, antibiotic markers and immunofluorescence techniques are helpful but have several limitations. Using the GUS reporter system, Vande Broek *et al.* (1993) studied the surface distribution of *A. brasilense* on developing wheat roots and showed that during the first days bacteria were mainly found in the root hair zones and at sites of lateral root emergence. The root elongation zone and root hairs remained devoid of bacteria, and strong colonisation was observed only in the absence of combined nitrogen. When plants were grown in rooting medium without combined nitrogen, bacteria were gradually enriched in

the lower part of the roots. This part corresponds to the microaerobic zone to which bacteria migrate or, to be more specific, multiply in zones of low oxygen concentration, allowing N_2 fixation to occur. The mechanisms of preferential colonisation at the sites of lateral emergence and in the root hair zones may involve bacterial chemotactic motility (Vande Broek *et al.*, 1998). Rhizobacteria may colonise the surface of the plants or/and tissues internal to the epidermis. Rhizobacteria that are able to colonise the interior of the plant and live within plant tissues are called endophytic bacteria. They are better placed to exploit carbon substrates supplied by the plant and grow within a low pO_2 environment, which is necessary for the expression of nitrogenase enzymatic activity, or to optimise plant-bacteria interaction. The status of PGPR as potential endophytic bacteria is not well known and colonisation of inner root tissues has to be examined with more attention using a confocal laser scanning microscope coupled with specific staining.

Potential Use of PGPR in Agriculture

Most studies have been conducted with *Azospirillum*-plant association on cereals and grasses and only a few other plant families have been investigated. The results of field inoculation are well documented for their lack of consistency. Most authors report the complexity of the phenomenon and poor understanding as the main explanations of inconsistent results. Early experiments (1974-1982) were designed to demonstrate promotion of growth by biological nitrogen fixation (BNF). Experimental objectives and designs were changed somewhat when it was found that plant growth promotion caused by *Azospirillum* inoculation is mainly due to promotion of root growth, leading to relatively more successes. The review presented by Okon and Labandera-Gonzales (1994) is based on results from field experiments conducted over 20 years throughout the world, and includes conclusions and recommendations on the feasibility of the agronomic use of PGPR. The picture emerging from the extensive data review is that of 60-70% successes with statistically significant increases in yield in the order of 5-30%. In five trials in which nitrogen nutrition was followed, improvement in N nutrition proved attributable to inoculation of maize with *Azospirillum lipoferum* strain CRT1 (Fages, 1994). In these experiments, the exogenous nitrogen from the fertiliser was better absorbed. The results of experiments with ^{15}N gave indirect confirmation of this nutritional improvement, since inoculation led to increase in several traits of plant growth, which accords with increase in mineral nutrition. Therefore, nitrogenous fertiliser was better absorbed by inoculated plants than by controls. The non-used part of N, which is susceptible to leaching, gets reduced and consequently the risks of pollution of groundwater by nitrates are also reduced. Plants with high dry matter content are able to assimilate much more nitrogen, which could be useful in soil depletion and preclusion of lixiviation. In the experiment carried out by Fages (1994), improvement in plant mineral nutrition seems to be

linked with a better root development promoted by the rhizospheric bacterium *A. lipoferum* strain CRT1. Many other pertinent remarks are found in the paper by Okon and Labandera-Gonzales (1994), who point out the possibility of reducing the factors causing failures in plant inoculation with rhizobacteria. A well-focused strategy taking into account all the possible reasons for failures and well adapted to the specific experimental conditions can ensure satisfactory results. Successful inoculation experiments appear to be those in which researchers pay particular attention to the optimal number of cells of bacteria in the inoculant, using appropriate inoculation methodology whereby the optimal number of cells remains viable and available to colonise roots. This statement ought to lead to a shift in the research programme of teams involved in PGPR-plant association studies in the field. Okon and Labandera-Gonzales also suggest that a well-focused strategy of field experimentation could demonstrate an acceptable consistency of agronomic results. For instance, the authors recommend water status of plants, percentage emergence and plant ontogeny as criteria for evaluation and interpretation of field results, taking into account the NPK nutrition. Moreover, before a field experiment is conducted, they recommend collection of information on crop history, microclimate, field soil conditions, residue management and yield levels.

Field experiments on a large scale involve a reliable inoculant with an acceptable level of technology for bacterial biomass production and formulation process enabling the bacterial inoculant to persist long enough to allow the growth-promotion effect. Application of the inoculant to introduce the rhizobacteria on the roots of the plant is a very significant step. Application method and formulation process are interlinked and determine the manner in which an accurate number of rhizobacteria can be delivered per seed or plant. The inoculant can be associated with seeds at sowing or can be added independently. For application in association with seeds, seeds and inoculant can be mixed just prior to sowing or in some cases precoated seeds can be used. In the latter instance the rhizobacteria must be in a dormant state since good survival is needed for several months. When application of the inoculant is independent of the seeds, microgranular material is used as a carrier which must be mixed with the inoculum itself. This method enables good control of the number of viable bacteria in the seed vicinity but implies a specific granular applicator for spreading the product in the furrow. Liquid inoculant application is another alternative for introducing the selected rhizobacteria on the root system of the plants.

CONCLUSION

Plant growth-promoting rhizobacteria are of considerable agronomic and scientific interest but many problems have to be solved before their large-scale application as biofertiliser. Diagnosis of a successful interaction is only

possible through analysis of yield parameters, such as dry weight of shoots and roots, nitrogen content and mineral content at the end of the experiment. The mechanisms of PGPR-plant root interaction and the basis of the beneficial effect have not been well understood. The ability of rhizobacteria to colonise the plant root system is difficult to accurately predict in natural conditions and depends on bacterial factors, soil properties and plant species or genotypes. Nitrogen-fixing bacteria have some nutritional advantages compared with other bacteria in the soil and root vicinity. Therefore, under conditions of strong competition for root excretion serving as the substrate for microbial growth, nitrogen-fixing bacteria may be able to colonise the plant root system more intensively and are probably still among the best candidates for modifying plant physiology. Changes in root morphology and root functions as mineral uptake or other modifications in plant gene expression have to be more clearly identified at an early stage. Specific physiological changes take place in plant roots inoculated with rhizobacteria just 24 hours after inoculation (Bertrand, 1997). This test could be used as a diagnostic tool of efficiency in promoting plant growth stimulation.

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