Chapter 1

Biofertilizers: Present and future use of transgenic micro-organisms

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Biofertilizers are living microbial preparations which enhance or promote plant growth, relatively to a control without inoculation. A huge amount of research literature has been produced in the last 20 years concerning plant growth-promoting rhizobacteria (PGPR) related subjects, describing different micro-organisms acting on different plants, and proposing different mechanisms to explain the plant growth promotion effect. However, we still do not know which of the different in vitro *mechanisms of biofertilizer action are responsible for the positive effects in the field. Biofertilizer technology has significantly developed in the market. The nature of multiple mechanisms discovered for PGPR actions and the possibility of genetically modifying a particular strain concerning a particular plant growth-promoting activity suggest that the use of genetically modified organisms such as biofertilizers will be an area of multiple and diverse possibilities of action in the near future. The study of the microbial ecology of this scenario and its dynamics will certainly improve the development of biofertilizer technology for the future of agriculture.*

Introduction

One of the big challenges for the future of humanity is to produce enough food in a sustainable way. Another big challenge is to produce bio-fuels to replace those non-renewable types of fuels for which resources will be exhausted some day. Crop plants play a key role for solving both of these challenges. Besides water, crop production is limited by the availability of the main nutrients in soil, such as nitrogen (N) and phosphorus (P). Soil micro-organisms are key elements in biogeochemical cycles of elements on our planet (Buscot and Varma, 2010).

From an evolutionary point of view, many plant/micro-organisms interactions have been selected which produced mutual benefits for the interacting organisms. Plants are primary biomass producers through photosynthesis and those photosynthates can be partially released into the soil via root exudates or via root and/or plant debris degradation. In this way, soil organic matter is increased and can be used by heterotrophic organisms as substrates to grow. It is reasonable to think that some microbes have succeeded in the history of evolution because of their capacities to improve plant growth, to assure their own source of food or substrates needed to grow. Most of these kinds of microbes live in the rhizosphere, the part of the soil which is influenced by the release of substances from the plant (Dessaux et al., 2010).

From a utilitarian point of view, these kinds of micro-organisms can be used to improve plant growth to assure food production. If these micro-organisms facilitate plant nutrition, their action would be valuable in terms of sustainability of the processes, because it would diminish the need for chemical fertilizers, whose production depends on non-renewable energy sources.

Taking all of these ideas into account, biofertilizers are defined as industrial products based on culturable micro-organisms that were isolated from the soil or rhizosphere of plants and which have been proven capable of modifying, and improving, plant development through a collection of different mechanisms of action. A product is characterised as a biofertilizer following an experimental test where the behaviour of a plant inoculated with a suspension containing a huge amount of cells of a particular micro-organism is compared to a control situation where the plant grows without the addition of this micro-organism. This experimental test can be performed either *in vitro* or *in vivo*. *In vitro* means growing plants hydroponically or using a controlled substrate, in pots in growth chambers or in greenhouses. *In vivo* means that the test is performed in soil, either in greenhouses or in field conditions. *In vivo* results may differ from *in vitro* results because the microbial background is different, and it is almost impossible to verify and compare the microbial background in soils with experimental *in vitro* conditions, simply because we still do not know precisely how to characterise the total microbial diversity existing in soil. Culturable micro-organisms are about 1% of the total existing micro-organisms in soil (Staley and Kanopa, 1985; Torsvik and Ovreas, 2002). So, regardless of the characterisation result of a micro-organism as a biofertilizer after different *in vitro* tests, the biofertilizer activity should be proven in soil, in field conditions, because the plant microbe interaction must function in the presence, and influence, of the huge diversity of other micro-organisms living in soil.

There are different modes of interactions between biofertilizers and plants (Gray and Smith, 2005), considering the degree of association between micro-organisms with plant roots in a gradient of root proximity and intimacy as follows:

1. micro-organisms living in the soil near the root, utilising nitrogen and carbon metabolites leaking from the root (rhizosphere)

2. micro-organisms colonising the rhizoplane (root surface)

3. micro-organisms colonising the root tissue inhabiting intercellular spaces (endophytes)

4. micro-organisms living inside cells in specialised root structures or nodules (symbionts).

Cases 1-3 do not induce any particular root structure and the micro-organisms are considered to be in a looser associative interaction with the plant compared to a more complex integrated association including the development of specialised root structures as in the last case of symbiotic associations. In all cases, biofertilizers should reach and colonise the rhizosphere to act on the plant through interaction with its root. It has been shown that root colonisation is part of the mechanism needed to produce plant growth promotion (Lugtenberg and Kamilova, 2009).

Besides the degree of association with the root tissue, at least two different modes of action can be recognised in biofertilizers' activity: direct and indirect mechanisms. Direct mechanisms imply the supply of a nutrient or the release of microbial substances which enhance plant growth or development. Indirect mechanisms are those which suppress or inhibit a deleterious situation for regular plant development, as for instance, a disease caused by a pathogen (Vessey, 2003; Glick et al., 1999).

For study purposes and organisation of the available knowledge on this subject, considering the different scenarios of plant-microbe interactions and the different mechanisms of plant growth promotion, different definitions are used to organise the concept of biofertilizer:

1. Free-living/non-symbiotic micro-organisms considered to be plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). These biofertilizers are also considered extracellular (Gray and Smith, 2005). To clarify different kinds of PGPR, different authors have proposed different definitions which try to separate them according to their mode of action:

    −   Plant growth-promoting bacteria (PGPB). These microbes promote direct plant growth by enhancing different mineral nutrition or by regulating plant development implying a phytohormone-like way (Bashan and de-Bashan, 2010; Verma et al., 2010).

    −   Biocontrol PGPB. These microbes are mainly antagonists to different plant pathogens. They improve indirectly plant growth by releasing the disease state of the plant (Bashan and Holguin, 1997). This particular group of micro-organisms has received some attention in the last years because of their economic implications (see Chapter 2).

    −   Plant stress homeostasis-regulating bacteria (PSHB). This group of micro-organisms was quite recently proposed to highlight cases were the plant growth promotion takes place within an abiotic stress condition (i.e. water stress, salt stress) (Cassan et al., 2009; Bashan and de-Bashan, 2010)

2. Micro-symbionts or intracellular plant growth-promoting micro-organisms, as defined by Gray and Smith (2005). These associations are more visible in the

plant because they induce specialised root structures where the plant microbe interaction takes place. All of these interactions are at least important for a main nutrient supply for the plant (nitrogen or phosphorus), but other positive concurrent mechanisms for plant growth promotion cannot be disregarded as part of the activity of these micro-organisms:

− N$_2$-fixing rhizobacteria:

❖ rhizobia-legume simbiosis

❖ *Frankia*-actinorhizal plant simbiosis.

− Micorriza fungi:

❖ arbuscular mycorriza fungi (AMF)

❖ ectomycorrhyza fungi (EMF).

What is new about this since the previous OECD consensus document dedicated to biofertilizers, published in 1995? Back then, 48 out of 51 pages referred to symbiotic biofertilizers, describing mostly rhizobia, *Frankia* and mycorrhiza, which are symbiotic cases. The remaining three pages of the document referred to free-living micro-organisms as "future biofertilizers". Since 1995, many articles have been published related to these new groups of micro-organisms generally named or referred to as PGPR. Different mechanisms of action have been described and are still a matter of intensive research. Most of this knowledge has been recently reviewed (Glick et al., 1999; Vessey, 2003; Bashan et al., 2004; Gray and Smith, 2005; Barriuso et al., 2008; Lugtenberg and Kamilova, 2009; Verma et al., 2010; Bashan and de-Bashan, 2010).

Symbiotic biofertilizers

As stated above, these symbiotic micro-organisms induce new structures in plants or infect the root tissue in quite a visible way under the microscope. It is because of this characteristic that these micro-organisms have been known for a long time, discovered by the end of 19th century (Werner, 1992). In many cases, the application of molecular biology tools allows the discovery of the genes and signals involved in the beneficial interaction between the micro-organism and the plant. The main symbioses concerning agricultural application as biofertilizers are considered below.

Rhizobia

The most advanced studied system corresponds to the nitrogen-fixing symbiosis between Gram negative bacteria generally called rhizobia, and leguminous plants, including many important crops for forage and food production (Werner, 1992). The molecules involved in the signal exchange between the bacteria and the plant which determines the recognition control the development of the infection and nodule structure in the root have been described in many model legumes (Schultze and Kondorosi, 1998). Different species of root nodule endophytes are included in the group of alpha-proteobacteria but some beta-proteobacteria were also found. The different genus and species inhabiting legumes root nodules are usually referred to as rhizobia. Within these endophytes genera are alpha-proteobacterias such as: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium (Ensifer)*, *Mezorrhizobium*, *Azorhizobium*, *Allorhizobium*, *Agrobacterium*; and beta-proteobacteria such as *Burkholderia*. The best model describing the interaction between rhizobia and legume roots includes flavonoids/isoflavnoids molecules released

by the plants which induce bacteria genes and consequently the synthesis of the LCO (lipo-chitin-oligosaccharides) molecules, which in turn control infection and nodule development in the root tissue (Schultze and Kondorosi, 1998; Madsen et al., 2010). Besides this Nod factor model characterised by the LCO molecules, a Nod factor independent nodulation of some legumes (Giraud et al., 2007) has been described. As a consequence of the knowledge developed over the last decades, the new generation of inoculants for legumes based on specific rhizobia strains has been improved by the use of new technologies, including the addition of those signals involved in the early interactions between the bacteria and the plant.

Mycorrhiza

The symbiosis between mycorrhiza fungi and the plant is not only the most ancient symbiosis for which we have fossil records, but it is also one of the most studied beneficial plant-microbe interactions (Smith and Read, 2008). Besides the kind of mycorrhizal association that the fungi establish with the plant, ecto- or endo-mycorrhiza, in all the cases the external fungi hyphae create a net outside the root that extend the exploration capacity of the plant root, improving the interaction of the plant with the soil, and the uptake of nutrients. It is well documented that mycorrhiza association improves water, nitrogen and phosphorus uptake by the plant, and probably other micronutrients.

Ectomycorrhiza (EMF) induce the formation of short modified roots where the fungus creates a net in the outer cell layers of the plant cortex without invading the plant cell but establishing a huge surface of plant-fungus interaction. The EMF are more specific in the association with plant species, and generally this symbiosis is present in woody plants. The EMF belong to culturable fungi species and there are different examples of biofertilizers based on this kind of micro-organism, whose market is orientated to tree and shrub production in nursery conditions.

Endomycorrhiza, also referred as arbuscular mycorrhiza fungi (AMF), is the most ancient documented symbiosis which is actually present in about 90% of plant species. Different fungi species are able to induce arbuscular mycorriza, the most abundant ones being fungi of the order Glomales. It has not been possible to cultivate any of the AMF in the lab. This fact is a very important limitation for the industrialisation of biofertilizers based on endomycorrizal fungi. Although the AMF do not appear to be active in conventional agriculture with tillage soil, they become important in soil conservative agricultural management, as direct sowing with no till or reduced till practices. Thus, from a sustainable point of view, the availability of pure AMF inoculum would be needed for good agriculture practices. The possibility of cultivating AMF strains in carrot root organ culture is good news and provides new possibilities in this area (Declerck et al., 2010). Quite recently, the chemical nature of the signals involved in the early recognition between the AMF and host plants has been described; the signals have been called Myc factors (Maillet et al., 2011). Curiously, the Myc factors are also lipochitinoligosacharide in nature as rhizobia Nod factors are. These signals appear to stimulate arbuscular mycorrhizal formation and also lateral root development. Most probably, these kind of signals will be part of the future generation of biofertilizers, as it was the case in rhizobia-Nod factors based inoculums.

Free-living or non-symbiotic biofertilizers

Since the description of PGPR by Kloepper and Schroth (1978), many different bacteria genera have been described as PGPR: *Pseudomonas*, *Azospirillum*, *Azotobacter*,

Gluconacetobacter, *Herbaspirillum*, *Bacillus*, *Burkholderia*, *Erwinia*, *Caulobacter*, *Azotobacter*, *Chromobacterium*, *Serratia*, *Micrococcus*, *Flavobacterium*, *Actinobacter*, *Enterobacter*, *Arthrobacter*, *Agrobacterium*, *Hyphomycrobium*, and fungus such as *Trichoderma*, among others (Bashan and de-Bashan 2010, Verma et al., 2010; Richardson and Simpson, 2011).

Many PGPR have been described as endophytic bacteria. It is not clear if the plant growth promotion effects are a consequence of plant-microbe interaction in the external part of the rhizosphere or if an endophytic state is necessary. Many different mechanisms have been claimed to be responsible for the plant growth promotion effect after *in vitro* experiments under controlled conditions (Glick et al., 1999). In some cases, the use of appropriate mutants helps in the definition of these mechanisms. But since different mechanisms are always present in a single strain, it is almost impossible to know which are the main mechanisms operating and driving the plant growth promotion. Irrespective of the real mechanisms operating in PGPR with a positive effect in field, the use of these micro-organisms has dramatically increased in recent years and will probably continue to grow because biofertilizers appear as a valuable opportunity for future sustainable agriculture. Many commercial products already exist which are based on *Pseudomonas* or *Azospirillum* strains in the market.

The different mechanisms operating in PGPR can be classified and discussed as: N (nitrogen) and P (phosphorus) nutrition effects, and plant root development and fitness mediated by phytohormones.

Nitrogen nutrition

One of the historically misleading cases in N nutrition mediated by PGPR was *Azospirillum* spp. Different strains of *Azospirillum* were initially characterised as free living diazotrophs able to fix nitrogen in micro-aerobic conditions (Döbereiner and Day 1976). In different experiments it was shown that inoculation of plants, mainly grasses, with *Azospirillum* enhanced plant growth, and this was initially attributed to N fixation/assimilation mediated by the bacteria. N balance measurements and *in situ* determination of acetylene reduction activity have shown that N fixation was not certainly the main reason of the plant growth stimulation mediated by *Azospirillum*, but an effect on root development and architecture appears to be the main mechanism responsible for the stimulatory effect (Bashan et al., 2004). Nevertheless using ^{15}N isotope techniques it was demonstrated that plants inoculated with diazotrophic PGPR (i.e. *Azospirillum*, *Herbaspirillum Gluconacetobacter*) benefited from N derived from fixation (Saxena and Tilak, 1998, Baldani and Baldani, 2005). The problem with N nutrition via free living diazotrophs is that fixed nitrogen is not released by the bacteria but assimilated for its own growth. The use of glutamine synthetase (GS) mutants of *Azospirillum* as plant inoculum improved plant growth compared to the parental strains (Van Dommelen et al., 2009).

Phosphorus nutrition

Micro-organisms are part of the soil phosphorus cycle and as such play an important role in mediating the availability of P to plants (Richardson and Simpson, 2011). Microbial enhancement of P availability is mediated by at least two different mechanisms: P solubilisation and P mineralisation.

Solubilisation of inorganic P from an insoluble chemical form is usually mediated by the ability of the micro-organism to acidify growth medium, to release organic anions such as citrate, gluconate, oxalate and succinate, and consequently to increase free phosphate in the medium or environment. This bacterial characteristic is usually tested in an agar plate medium with precipitated tricalcium phosphate which is clarified by the acid released from the bacterial colony. This microbial activity can also be measured and quantified in a liquid medium (Fernández et al., 2012). The ability to solubilise P in a culture medium is a potential activity and does not always guarantee biofertilizer activity in the field. Field experiments should be done with the amendment of insoluble P source to test if these bacteria can enhance P availability in field conditions and consequently improve plant growth, behaving as true biofertilizers.

Alternatively, P can be released from organic matter in the soil by mineralisation procedures mediated by enzymatic activities released by the micro-organism. Different enzymes have been characterised to mediate this activity such as phosphatases, phytases and phospholipases, which are key drivers in this transformation, independently of organic matter turn over. Again, the problem of the destiny of the released phosphorus appeared as in the case of fixed N; there would be competition between the bacteria and the plant for the released P. Thus, the final effect of these bacteria in plant growth promotion should be tested in field conditions. Since the availability of released P is independent of the plant species which can make use of it, the biofertilisation concerning P nutrition has been developed in different commercial products based on different bacteria and fungi species, orientated to a broad spectrum of plant species.

Phytohormones mediated mechanisms of plant growth promotion induced by micro-organisms

One of the most visible effects on plants after inoculation with PGPB is the huge development – and sometimes changes in the architecture – of the root of the plant. This general improvement of root growth, including root hairs development, is one of the characteristic phenotypes of the interaction plant-PGPB.

It is likely that water and mineral uptake is consequently improved because of the increase in the root system, although the specific mechanism is not completely clear. Changes in hormone balance, enhancement of proton efflux activity extrusion and modification in a wide range of related enzymatic activities would be part of the mechanisms behind this phenotype (Bashan and de-Bashan, 2010; Cohen et al., 2009). Most of the existing data is, however, descriptive.

Auxins

This general root improvement phenotype can be reproduced by replacing phytohormones with PGPB, and phytohormones-like substances have been detected in bacterial culture supernatants so it is likely that this phenotype is mediated by phytohormones synthesised by the bacteria (Costacurta and Vanderleyden, 1995). Auxin-related substances, such as indole acetic acid (IAA), appear to be involved in one of the

most important mechanisms regarding this general root development improvement. Nevertheless, bacterial production of IAA *in planta* has not yet been demonstrated. There are no IAA completely deficient mutants, but IAA attenuated mutants were ineffective as PGPB, compared to parental strains (Bashan and de-Bashan, 2010).

Gibberellins

Gibbelleric acids (GAs) are produced by some PGPB species *in vitro* and have also been shown *in planta* since those PGPB strains capable of producing GAs *in vitro* were able to complement GA-deficient mutant dwarf rice by inoculation (Bottini et al., 2004). PGPB producing GAs were also active in improving seed germination. *In vitro* results support the hypothesis that PGPB effect would be a combination of GA production and GA-glucoside/glucosyl ester deconjugation by the PGPB.

Cytokinins

The adenine-type cytokinins represented by kinetin, zeatin and 6-benzylaminopurine which occur in plants have also been produced in a defined culture medium by many PGPB (Strzelczyk et al., 1994). The role of citokinins in the promotion of root development is not clear, but cytokinin-producing PGPB stimulate nodulation in legumes when co-inoculated with rhizobia, and it was recently demonstrated that there is a Nod factor independent mechanism for infection and nodulation (Giraud et al., 2007), probably mediated by rhizobial cytokinin (see helper bacteria, below). This particular area deserves more attention in the future.

Ethylene/ACC deaminase

Ethylene is a plant hormone related to general plant responses when a stress condition appears, even if it is a very low stress situation (Glick, 2004). When this happens, the plant synthesises ethylene and stops its growth temporarily because of the regulatory effects of ethylene on different cell functions. 1-aminocyclopropane-1-carboxylate (ACC) is a precursor of ethylene synthesis. The enzyme ACC deaminase is present in some bacteria which can even use ACC as C (carbon) and N sources. When ACC deaminase is expressed by a rhizospheric bacteria root growth and development is enhanced, it is probably because of the elimination of the inhibitory concentrations of ethylene produced by the plant (Glick, 2004). This enzyme is not ubiquity present in bacteria and its activity is codified by a single gen *acdS*. The introduction of this gene from *Pseudomona putida* into other bacteria species confers plant growth-promoting functions to the recipient bacteria that were absent in the parental strain (Glick et al., 2007). This represents a potential biotechnological tool to improve micro-organisms to be used as biofertilizers.

Nitric oxide

Nitric oxide (NO), a plant regulator volatile phytohormone, is also produced by some PGPB as *Azospirillum* spp. (Molina-Favero et al., 2008). Bacterial NO is an intermediary in IAA-induced root development. NO can also mediate plant growth-promoting activity in *Azospirillum brasilense* Sp245 inducing morphological changes in tomato roots regardless of the full bacterial capacity for IAA synthesis.

Polyamines

Azospirillum spp. can produce different ployamines in culture (Perrig et al., 2007, Cassan et al., 2009). Cadaverine is synthesised by these bacteria from lysine mitigated osmotic stress in rice seedlings, based on improved water status and decreased production of ABA in inoculated seedlings (Cassan et al., 2009).

Helper bacteria

In the studies of plant microbe interaction which induced some kind of plant growth promotion, there are other cases that do not fit into the previous definitions but which can be considered as another kind of biofertilizer. That is the case of bacteria which improve a plant-microbe interaction as a third partner in the interaction. An example can be found in rhizospheric actinomycetes isolated from legumes or actinorhizal nitrogen-fixing nodules (Solans, 2007) which are able to stimulate nodulation, consequently nitrogen fixation in the plant, and finally plant growth (Solans et al., 2009). This tripartite plant-microbe interaction is not well known yet in terms of mechanisms, but clearly shows that biofertilizers can be improved by the use of more than one micro-organism at a time.

Conclusion

Although not all the different bacterial mechanisms that have been claimed to be responsible for the plant growth promotion phenomenon are present in a single strain, it is also true that each single strain usually shows more than one characteristic activity related to plant growth promotion. Thus, it has been almost impossible to prove with certainty the relevance of each and every mechanism described as plant growth-promoting activities in selected micro-organisms. This is especially true when the plant growth-promoting activity is tested in field conditions. Despite this uncertainty, the positive results are reproducible and no harmful effects have appeared. Thus, practical application of biofertilizers is increasing worldwide.

The nature of multiple mechanisms discovered for PGPR actions and the possibility of genetic modification of a particular strain to enhance its PGPR activity, suggest that the use of genetically modified organisms is not needed to implement this technology but could be a way to improve what can be found in nature.

In addition to all these descriptions which try to give an overview of the current state of the art in biofertilizers, it must be pointed out that nowadays the picture of soil microbial ecology is completely different from what it was when biofertilizers were discovered and began to be studied. Microbial soil ecology appears as a very complex and mostly unknown scenario where all these PGPR-plant interactions take place. The study of the soil microbial ecology and its dynamics will certainly improve the development of new and better biofertilizer technology for the future of agriculture. Since the same plant growth-promotion function or mechanism could be driven by many different bacteria or micro-organisms, this functional redundancy in soil microbial diversity may be managed in favour of plant development.

As this chapter has shown, the mechanisms that are at the basis of plant growth promotion by micro-organisms are beginning to be unravelled at the molecular level. This knowledge is already used for strain improvement by genetic modification, and there are several areas, e.g. introducing an ACC deaminase gene in PGPB strains which lack this
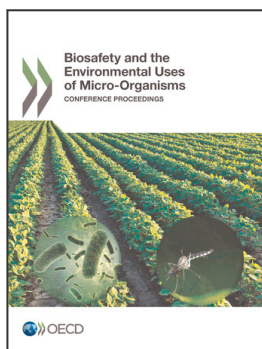
particular activity (Glick et al., 2007), creating overproducing IAA strains (Bashan and de-Bashan 2010), genetically modified strains which release the fixed ammonium (Van Dommelen et al., 2009), where important improvements of the potential for plant growth stimulation of bacterial strains may be achieved. Environmental risk assessment of the use of such strains will require a solid knowledge about the mechanisms behind plant growth stimulation. For instance, horizontal gene transfer of ACC deaminase genes in rhizospheric bacteria has been suggested (Hontzeas et al., 2005). It is clear that quite some new insights and knowledge have become available in this area since the previous OECD publication (OECD, 1995).

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