Chapter 8
Biotechnology of the Rhizosphere

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Abstract This chapter deals with the management of the rhizosphere as a living system, paying special attention to one of the three partners that define the rhizosphere: beneficial microorganisms (termed *PGPR* or the plant growth-promoting rhizosphere bacteria) that inhabit it. After that, several biotechnological approaches for management of the rhizosphere will be presented. These approaches relate to environment friendly agricultural practices, the production of high-quality foods with bioactive compounds (*phytonutrients*), and applications in the pharmaceutical industry.

The *rhizosphere* refers to the soil region that is subject to the influence of plant roots and their associated microorganisms. Among these microorganisms are plant growth-promoting rhizobacteria which are beneficial for plant health in many ways: by improving plant nutrition, protecting against other microorganisms, producing plant growth regulators, or enhancing plant secondary metabolic pathways that are directly related to a plant's defense. In some plant species, these secondary metabolites are useful to human health.

The biotechnology of the rhizosphere covers a wide array of applications that deal with sustainable agriculture (intensive or extensive): lowering of chemical inputs due to fertilizers and pesticides; improving crop productivity in saline and non-fertile soils; improvement of plant fitness for reforestation of degraded soils; and improvement in the bioactive levels of metabolites in medicinal plant species, among others. In this connection, the identification of *elicitors* (molecules that stimulate any of a number of defense responses in plants) appears to be an alternative to PGPR for unraveling limiting steps of secondary metabolism pathways.

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8.1 Introduction

The German agronomist Hiltner first defined the rhizosphere, at the end of the nineteenth century, as the "effect" of the roots of legumes on the surrounding soil, in terms of higher microbial activity, due to the organic matter released by the roots (Lynch, 1990). Until the end of the twentieth century, this "effect" was not considered to be an ecosystem in which the three components (plant, soil, and microorganisms) define a unique environment (Barriuso et al., 2008a). This environment changes depending on the conditions set up by the three components. Therefore, a deep knowledge of the interactions between the plant, the soil, and the microorganisms is vital to our understanding of how this complex rhizosphere system operates.

In this context, a second concept that needs to be addressed here is what we shall term biotechnology of the rhizosphere (Fig. 8.1).

Among the three components of the interaction shown in Fig. 8.1, microorganisms appear as the easiest element to manipulate, since we will usually select the plant of interest and the soil to work with. Microorganisms that inhabit the rhizosphere play a key role in plant physiology by affecting either directly or indirectly the plant's metabolism. These bacteria may increase nutrient availability in the soil, which will be reflected in better growth of the plant (indirect mechanisms), or may affect the plant's hormonal balance or its secondary metabolism (direct mechanisms) (Ramos Solano et al., 2008a). When secondary metabolism is affected, the plant's defense against pathogen or insect attack may be improved for better fitness. At the same time, in medicinal plant species, levels of phytopharmaceuticals are altered. In this case, either known metabolites increase or even new molecules may appear (Poulev et al., 2003). This role involves not only the direct effect of a single bacterial strain but also that of the molecular dialogue established among soil microorganisms and between microorganisms and the plant (Barriuso et al., 2008c).

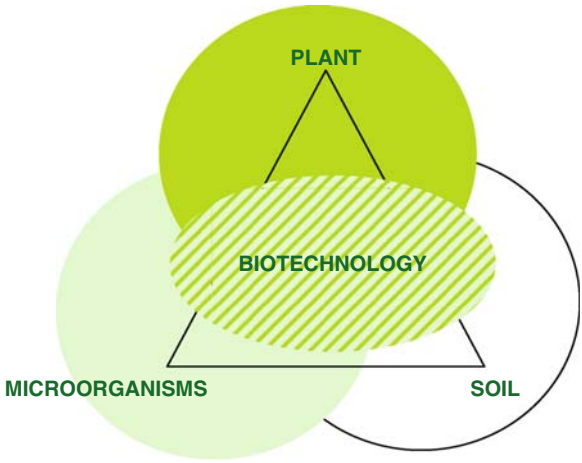

Fig. 8.1 Biotechnology of the *rhizosphere*. The *rhizosphere* is defined by the interaction of three components: plant, soil, and microorganisms. Biotechnology of the rhizosphere refers to management of any of the three factors and/or their interactions in order to obtain a certain effect

A thorough understanding of the PGPR action mechanisms is fundamental to manipulating the rhizosphere in order to maximize the processes within the system that strongly influence plant productivity. Therefore, the first goal of this chapter will be to examine rhizosphere microorganisms and then to explore their different biotechnological applications.

8.2 Plant Growth-Promoting Rhizobacteria (PGPR)

A large number of macroscopic organisms and microorganisms such as bacteria, fungi, protozoa, and algae coexist in the rhizosphere. The most abundant are bacteria. Plants release organic compounds via root exudates, which selectively attract beneficial bacteria (Lynch, 1990), creating a very selective low-diversity environment (Marilley and Aragno, 1999; Lucas García et al., 2001; Barriuso et al., 2005). Bacteria inhabiting the rhizosphere beneficial to plants are called *PGPR* (*Plant Growth-Promoting Rhizobacteria*) (Kloepper et al., 1980a). The rhizosphere of wild plant species appears to be the best source from which to isolate PGPR due to the co-evolution processes that have taken place over time (Lucas García et al., 2001; Gutiérrez Mañero et al., 2003; Barriuso et al., 2005; Ramos Solano et al., 2007).

PGPR have been reported as members of several genera including *Azotobacter*, *Acetobacter*, *Azospirillum*, *Burkholderia*, *Pseudomonas*, and *Bacillus* (Arshad and Frankenberger, 1998). The positive effect of PGPR occurs through various mechanisms.

Mechanisms used by PGPR have traditionally been grouped into direct and indirect mechanisms (for a recent review, see Ramos Solano et al., 2008a). Although the difference between them is not always obvious, *indirect mechanisms*, as a general rule, are those that happen outside the plant, while *direct mechanisms*are those that occur within the plant and directly affect the plant's metabolism. This means that the latter require the participation of the plant's defensive metabolic processes, which transduce the signal sent from the bacteria influencing the plant. Accordingly, indirect mechanisms are usually related to nutrient-related traits or defense against other microorganisms outside the plant, while direct mechanisms include those that affect the balance of plant growth regulators, either creating an outbound gradient from the roots to the soil (Glick et al., 1998) or because the microorganisms themselves release growth regulators that are integrated into the plant, leading to an improvement in its adaptative capacity (Gutiérrez Mañero et al., 1996, 2001). Two important phenomena are included in this group: systemic induction of secondary metabolism related to defense against plant pathogens and protection against high-salinity conditions (Barriuso et al., 2008b).

However, the existence of microorganisms able to prevent diseases from occurring in plants without the plant's direct participation is also known. This occurs by systems such as niche exclusion or pathogen-inhibiting substance production. When the physical contact of the pathogen and the protecting microorganism is required, it is known as *biocontrol* (Bloomberg and Lugtenberg, 2001; Compant et al., 2005).

A short review of the most relevant mechanisms follows and will be integrated into the subsequent case studies section later in this chapter.

8.2.1 PGPR That Utilize Indirect Mechanisms

The list of *indirect mechanisms* used by PGPR is substantial. A number of reports in the literature illustrate these types of mechanisms, and some are quoted herewith. Two groups can be devised, depending whether they are related to improvement of plant nutrition or pertain to pathogen performance. The first group includes (1) free nitrogen fixation, (2) siderophore production, and (3) phosphate solubilization. The second group includes (1) hydrolysis of molecules released by pathogens (e.g., Toyoda and Utsumi, 1991 reported the ability of two strains, *Pseudomonas cepacia* and *Pseudomonas solanacearum*, that are able to break down fusaric acid, a compound responsible for root rot caused by the fungus *Fusarium*); (2) synthesis of enzymes that are able to hydrolyze fungal cell walls (Lim et al., 1991); and (3) synthesis of cyanhydric acid (Voisard et al., 1989).

In addition, improvement of symbiotic relationships with rhizobia and mycorrhizae has been reported (Duponnois and Plenchette, 2003; Founoune et al., 2002; Garbaye, 1994; Marek-Kozackuk and Skorupska, 2001; Lucas García et al., 2004; Barriuso et al., 2008d), although further research will demonstrate whether these are direct or indirect.

Among indirect mechanisms, the most relevant for agricultural purposes are those involving nutrient mobilization (e.g., free nitrogen fixation, siderophore production, and phosphate solubilization). Such nutrient mobilization results in a lowering of chemical inputs to the environment, since the amount of chemical fertilizers necessary to achieve good crop yields would be lower. Interestingly, and for a proper and successful handling of this type of PGPR, it should be taken into account that there is increasing evidence that nutrient-related traits are inducible when the environmental conditions require such a need (Rainey, 1999). Otherwise, it may be the reason for the lack of success of some field inoculations (Ramos Solano et al., 2007). Moreover, if used appropriately, especially in low-fertility soils, these could be turned into better soils by increasing the culturable soil surface, which is one of the limiting factors currently needed to palliate world famine.

A short description of nutrient-related traits follows.

8.2.1.1 Free Nitrogen Fixation

These types of nitrogen-fixing bacteria were the first PGPR assayed to improve plant growth, especially crop productivity. The first report of these bacteria appeared before World War II, when they were widely used on cereal fields in the Soviet Union (Bashan and Levanony, 1990). They are free-living organisms able to fix nitrogen that inhabit the rhizosphere but *do not establish a symbiosis* with the plant. Although they do not penetrate the plant's tissues, a very close relationship is established; these bacteria live so close to the roots that the atmospheric nitrogen fixed

and not used by the bacteria is taken up by the plant, forming an extra supply of nitrogen. This relationship is described as an unspecific and "loose" symbiosis. Biological nitrogen fixation is a high-cost process in terms of energy. Bacterial strains able to perform this process do so to fulfill their needs, and thus, little nitrogen is left for the plant's use. However, difficulties may be overcome by biotechnological approaches based on genetic manipulations and other strategies to improve colonization capacities.

However, growth promotion caused by nitrogen-fixing PGPR was erroneously attributed to nitrogen fixation for many years, until the use of nitrogen isotopes occurred. This technique showed that the benefits of free nitrogen-fixing bacteria are due more to the production of plant growth regulators than to nitrogen fixation (Baldini, 1997). This kind of production of plant growth regulators is discussed later.

8.2.1.2 Production of Siderophores

Iron is an essential nutrient for plants. Iron deficiency is manifested in severe metabolic alterations due to its role as a cofactor for a number of enzymes essential to important physiological processes such as respiration, photosynthesis, and nitrogen fixation. Iron is quite abundant in soils, but it is frequently unavailable for the plant or soil microorganisms, since the predominant chemical species is Fe^{3+}, the oxidized form that reacts to form insoluble oxides and hydroxides, inaccessible to plants or microorganisms.

Plants have developed two strategies for efficient iron absorption. The first one consists of releasing organic compounds able to chelate iron, making it soluble; iron diffuses toward the plant where it is reduced and absorbed by means of an enzymatic system present in the cell membrane. The second strategy consists of absorbing the complex formed by the organic compound and Fe^{2+}, where the iron is reduced inside the plant and readily absorbed. Some rhizosphere bacteria are able to release iron-chelating molecules to the rhizosphere and, hence, serve the same function as in plants (Kloepper et al., 1980b).

Siderophores are low molecular weight compounds, usually below 1 kDa, which contain functional groups capable of binding iron in a reversible way. The most frequent groups are hydroximates and catechols, in which the distances among the groups involved are optimal to bind iron. Siderophore concentration in soil is around 10^{-30} M.

Siderophore-producing bacteria usually belong to the genus *Pseudomonas*, the most frequent being *Pseudomonas fluorescens*, which release the siderophores, pyochelin and pyoverdine. Rhizosphere bacteria release these compounds to increase their competitive potential, since these substances have antibiotic activity and improve iron nutrition for the plant (Glick, 1995).

Siderophore-producing rhizobacteria improve plant health at various levels: they improve iron nutrition, inhibit the growth of other microorganisms with their antibiotic molecules, and hinder the growth of pathogens by limiting the iron available for the pathogen, generally fungi, which are unable to absorb the iron–siderophore complex. Hence, siderophore-producing bacteria could be released to improve iron

nutrition at the same time that certain pathogens are controlled, resulting in lower chemical inputs due to pesticides and fertilizers.

8.2.1.3 Phosphate Solubilization

After nitrogen, phosphorous is the most limiting nutrient for plants. However, phosphorous reserves, although abundant, are not available in forms suitable for plants. Plants are only able to absorb the soluble forms, namely, monobasic and dibasic phosphates. Besides inorganic forms of phosphorous in soil, the phosphorous present in organic matter is of considerable importance. The organic forms of phosphorous are estimated to be between 30 and 50% of the total phosphorous in the soil. This reservoir can be mineralized by microorganisms, making it available to the plant as soluble phosphates. There are many bacteria from different genera that are able to solubilize phosphate. These include *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, *Chryseobacterium*, and *Erwinia*. Bacteria use two mechanisms to solubilize phosphate: (1) releasing organic acids that mobilize phosphorous due to ionic interactions with the cations of the phosphate salt and (2) releasing phosphatases responsible for releasing phosphate groups bound to organic matter. Most of these bacteria are able to solubilize the Ca–P complex, and there are others which operate in the Fe–P, Mn–P, and Al–P complexes. Generally, these mechanisms are more efficient in basic soils.

Results with PGPR able to solubilize phosphate are sometimes erratic, probably due to soil composition, given the inducibility of nutrient-related traits. In fact, in order to have a good performance, they would have to be inoculated in soils with a phosphorous deficit and stored in insoluble forms. Hence, inoculations of these types of PGPR sometimes improve plant growth and sometimes they are completely inefficient. Without doubt, knowledge of their mechanisms and ecology in the rhizosphere will improve their use in sustainable agriculture (Gyaneshwar et al., 2002).

8.2.2 PGPR Using Direct Mechanisms

*Direct mechanisms*are those that occur inside the plant and directly affect the plant's metabolism (Ramos Solano et al., 2008a) by involving the plant's defensive metabolic processes, which transduce the signal sent from the bacteria that influence the plant. Plant growth regulators can be considered as participants in the principal PGPR mechanism, together with the induction of systemic resistance (ISR), which has in recent years become an important issue. Both involve the existence of bacterial eliciting molecules, receptor binding, and further signal transduction. When bacteria release a plant growth regulator, all three stages are known, because this process is the same in plants and bacteria. However, this is not the case for induction of systemic resistance, in which the eliciting molecules, the receptor, and the signal transduction mechanism, as a general rule, are still unknown.

8.2.2.1 PGPR That Modify Plant Growth Regulator Levels

Plant growth regulator production by bacteria was first described more than 40 years ago. This was determined in the 1960s using the biological assays then available. Nowadays, using modern techniques, it has been demonstrated that the production of plant growth regulators such as auxins and ethylene by bacteria is a common trait (Bent et al., 2001). Others, such as cytokinins, are less common, while gibberellins in high concentrations have only been described for two strains of the genus *Bacillus*, isolated in the rhizosphere of *Alnus glutinosa* (Gutiérrez Mañero et al., 2001), the amounts being 1,000 times higher than those reported for *Rhizobium* that is involved in forming the nodule.

Modification of a plant's physiology by plant growth regulator production is a very important mechanism, not only because it alters the principal mechanism of growth regulation and cell differentiation in the plant but also because it is based on the evolutionary development of common metabolic pathways in plants and bacteria. This implies interesting co-evolution aspects. Biosynthetic pathways of plant growth regulators share many steps with the classical secondary metabolism pathways. This suggests a common ancestor, which in the course of evolution has produced either a large diversion in the function, conserving the genetic homology, or the function has remained the same, but there has been a large genetic divergence. This is evident in the phenolic compound biosynthesis pathway (shikimic acid pathway), which is shared by both plants and microorganisms. It is essential for synthesis of amino acids such as tryptophan, the precursor in auxin biosynthesis. The same occurs in the biosynthetic pathway of terpenes, gibberellin precursors. Therefore, the existence of common biosynthetic pathways and metabolic products implies the possibility of creating a parallel evolutionary connection between plants and microorganisms. Furthermore, it is striking that secondary metabolites synthesized by plants for defense also target some human receptors that affect human physiology, making the interest in these compounds even more interesting.

The production and release of plant growth regulators by bacteria cause an alteration in the endogenous levels of plant growth regulators. This is dependent on several factors, including (1) plant growth regulator concentration; (2) the proximity of the bacteria to the root surface; (3) the ability of the growth regulator to diffuse in soil and be transported across plant cell walls to the interior compartments of the cells; and (4) the competitiveness of the bacteria to colonize and survive in areas where there is high root exudation.

Based on the above discussion, we see that the effect of bacteria on the plant growth regulators' balance depends on many factors, and because of this, results with these different types of PGPR may vary. Moreover, a PGPR producing more than one type of plant growth regulator can cause a synergistic effect when their action is coupled. The next logical points to consider here are the main physiological functions of each growth regulator. A short description for each follows.

The production of hormones such as *gibberellins* or *cytokinins* has been reported for a small number of bacteria able to produce these plant growth regulators (Timmusk et al., 1999; de Salomone et al., 2001). Cytokinins are known to induce

cell division (Salisbury, 1994) and have recently been reported in free-living bacteria (Arkhipova et al., 2007). Concerning gibberellins, there is little information regarding microorganisms that produce this type of plant growth regulator. However, it is known that symbiotic bacteria that form nodules in the plant to fix nitrogen (Rhizobia) are able to produce gibberellins, auxins, and cytokinins in very low concentrations when the nodule is forming at the time of high cell duplication rate (Atzorn et al., 1988). However, the production of gibberellins by PGPR is rare, with only two described strains able to produce gibberellins in relevant concentrations: *Bacillus pumilus* and *Bacillus licheniformis* (Gutiérrez Mañero et al., 2001).

Auxins are derived from tryptophan metabolism, and their effects depend on the concentration, the organ affected, and the physiological status of the plant. Auxins synthesized by the plant and the microorganisms only differ in the biosynthetic pathway, depending on the plant and/or the microorganisms. More than 80% of soil bacteria in the rhizosphere are capable of producing auxins. Thus, the potential of these microorganisms to affect the endogenous levels of this regulator, and therefore their effects on plant growth, is remarkable.

The reason there are so many bacteria in the rhizosphere that are able to produce auxins is still unknown. Some authors suggest that these bacteria have a tryptophan-related metabolism and that auxin biosynthesis represents a detoxification mechanism (Bar and Okon, 1992). Other authors propose that auxins have some cellular function because a clear relationship has been observed between auxin and cyclic AMP (adenosine monophosphate) levels, which regulate many metabolic processes (Katsy, 1997). However, the anthropomorphic view of this fact could be correct, namely, that auxin synthesis improves plant growth that results in more exudation and more nutrients for rhizobacteria. This hypothesis explains a mutualistic beneficial association between rhizospheric microorganisms and the plant. The plant controls the energy flux in the system because it has more genetic information and contributes most of the organic matter to the rhizosphere.

Auxins released by rhizobacteria mainly affect the root system, increasing its size and weight, branching number, and the surface area in contact with soil. All of these changes lead to an increase in the ability of roots to extract nutrients from the soil, therefore improving plant nutrition and growth capacity (Gutiérrez Mañero et al., 1996). Another important result of inoculation with auxin-producing bacteria is the formation of adventitious roots, which are derived from the stem. The auxins induce dedifferentiation of the stem tissues to dedifferentiate as root tissue. All the above effects can vary considerably depending on the auxin levels that reach the root system, including an excess, which could be inhibitory. In order to explain these inhibitory auxin effects, the relationship of auxin with ethylene has to be considered.

Ethylene is another growth regulator whose levels alter PGPR, in turn affecting physiological processes in the plant. It primarily functions in regulating plant development processes, including seed germination, root growth, leaf abscission, fruit development and ripening, as well as defense systems and stress responses. Factors such as light, temperature, salinity, pathogen attack, and nutrition can cause marked variations in ethylene levels. The influence of abiotic factors in ethylene

levels was deduced some time before biotic factors were discovered (Abeles et al., 1992; Morgan and Drew, 1997).

As ethylene levels decrease, root systems increase their growth, with the benefits already mentioned. Using PGPR to reduce ethylene levels in the plant could be an interesting method to improve certain physiological processes in the plant. Ethylene biosynthesis starts in the methionine cycle; one aminocyclopropanecarboxylic acid molecule (ACC) results from each turn of the cycle. The enzyme responsible for ACC production is ACC synthase, whose expression level and activity are regulated by a large number of signals such as auxin, ethylene, and environmental factors. The ACC is the substrate for ACC oxidase, also called ethylene-forming enzyme (EFE). This enzyme has been cloned from numerous species and belongs to a multigenic family which produces different types of ACC oxidases depending on the plant organ and development state.

The model proposed for ethylene regulation in the plant by PGPR is based on the ability of some bacteria to degrade ACC, the direct precursor of ethylene (Glick et al., 1994a). The degradation of this compound creates an ACC concentration gradient outbound, favoring its exudation and, hence, a reduction of the ethylene level inside. This, in combination with auxins that may be produced by the same microorganism, has a considerable impact on important physiological processes, such as root system development, since the bacterial ACC deaminase competes with the plant's ACC oxidase. This ACC deaminase enzyme has been isolated and identified in several bacterial and fungal genera, all having the ability to use ACC as the sole nitrogen source. Curiously, no microorganism has yet been found that is able to form ethylene from ACC (Glick et al., 1994b). Since ethylene and auxins are two related types of growth regulators and since the balance between them is essential for the formation of new roots, some effects attributed to auxin-producing bacteria are actually due to ACC degradation.

PGPR that reduce ethylene levels in plants are also able to improve nodule formation in legumes and mycorrhizae formation in many other types of plants. A temporary reduction of ethylene in the earlier stages of either of these processes is beneficial.

Case study: The aim of this case study involving two separate studies (Gutiérrez Mañero et al., 1996, 2001) is to highlight the synergistic effects of bacterial strains producing two types of plant growth regulators.

These bacteria were isolated from the rhizosphere of *A. glutinosa* and production of IAA-like compounds in the culture media was demonstrated by bioassay. This bioassay was set up by adding bacteria cultures media free of bacteria to alder seedlings in two different concentrations. When a bacterial strain tested positive for enhancement of shoot and root growth, the results were plotted against data for plants that were grown on media containing increasing concentrations of IAA (Gutiérrez Mañero et al., 1996). However, addition of synthetic IAA to plants did not reproduce exactly the same effects as obtained for compounds released by bacteria, when their growth parameters were studied. Higher shoot surface suggested the presence of gibberellin-type compounds. Hence, a second study was carried out to detect these compounds. First, a bioassay was performed and second, identification

of putative compounds by HRGC-MS was employed. Bacterial culture media free of bacteria were concentrated and added to the shoot tips of young, dwarf alder seedlings; a control with GA_3 was also used. The same bacterial medium that was free of bacteria was used for HRGC-MS identification. These strains have shown a capacity to produce large quantities of gibberellins (GA_1, GA_3, GA_4, and GA_{20}) in vitro. The gibberellins were identified by HRGC-MS, and the amounts detected reached 200 $ng \cdot mL^{-1}$, GA_1 being the most abundant (130–150 $ng \cdot mL^{-1}$). These amounts were 1,000 times higher than for any other example of fungal or bacterial gibberellin production reported. Furthermore, the combination of gibberellins produced caused a balanced physiological effect in the plant opposite to the effects of GA_3 alone. This resulted in excessively long stems with pale yellow leaves. The suggested reason for the pronounced effect of gibberellins released by the PGPR present in the rhizosphere is that these hormones can be translocated from the roots to the aerial parts of the plant. The effects in the aerial part are notable, and even more so, when the rhizobacteria also produce auxins that stimulate growth of the root system. This enhances the nutrient supply to the sink generated in the aerial parts.

Based on these results, rhizobacteria able to release plant growth regulators can be formulated in a *biofertilizer*, with its intended use being to strengthen plant growth without any chemical input to the system.

8.2.2.2 PGPR That Induce Systemic Resistance (ISR)

At the beginning of the 1990s, Van Peer et al. (1991) and Wei et al. (1991) made an important discovery about plant defense mechanisms and productivity. These investigators found that certain non-pathogenic bacteria were able to prevent a pathogen attack before the pathogen reached the plant. The difference with biocontrol is that the beneficial bacteria do not interact physically with the pathogen but instead trigger a response in the plant which is effective against subsequent attacks by a pathogen. This response is systemic; that is, the bacteria interact with the plant in a restricted area, but the response extends to the whole plant. This response is mediated by metabolic changes that are not evident at first glance. As a matter of fact, *priming* or *biopriming* is the physiological state of a plant that is systemically induced by non-pathogenic bacteria against subsequent pathogen attack; but, the effect is not detected until pathogen challenge occurs (Conrath et al., 2002). Since energetic metabolism is diverted to secondary metabolism, this physiological state is usually coupled to lower growth rates as compared to non-primed controls (van Hulten et al., 2006). For the protection to be effective, an interval is necessary between the PGPR–plant contact and the pathogen attack in order for the expression of the plant genes that are involved in the defense. This mechanism was first known as "rhizobacteria-mediated induced systemic resistance" (Liu et al., 1995), but it is now termed "induced systemic resistance" (*ISR*) (van Loon et al., 1998). ISR was reported in the plant–pathogen-beneficial bacteria model, *Arabidopsis thaliana–Pseudomonas syringae DC3000–P. fluorescens WSC417r*. Here, the defensive response induced by *P. fluorescens* WSC417r in *A. thaliana* against *P. syringae* DC3000 is mediated by JA (jasmonic acid) and ethylene. Since then, it has been described in many plant species, including bean,

tobacco, tomato, and radish, with different PGPR and pathogens, and an increasing number of signal transduction pathways. This finding is fundamental because it proposes an "immune" *response* in the plant, raising the possibility of "vaccination" for the plant.

The plant can also acquire immunity after a pathogen attack. This response has been described before the ISR. The acquisition of resistance by the plant after a pathogen attack, causing little damage or localized necrosis in response to a further pathogen attack, has been known for many years. The phenomenon is called *systemic acquired resistance* (*SAR*) (Ryals et al., 1996). During a pathogen attack, *reactive oxygen species* (*ROS*) are produced in necrotic areas, causing tissue death. If the plant survives the challenge, it remains protected for life.

In *A. thaliana*, the SAR and ISR responses are regulated by distinctly different pathways. SAR is associated with an increase in salicylic acid levels and the translation of an ankyrin-type protein called NPR1, located in the nucleus, which induces the transcription of the pathogenetic-related (PR) genes. These genes codify the PR proteins that are responsible for systemic resistance in the plant (Lawton et al., 1991; Uknes et al., 1993). In the ISR response, salicylic acid levels are not altered, but the response is mediated by other two growth regulators, namely, ethylene and jasmonic acid, which act as signal transductors and not as stress hormones. In ISR, the NPR1 protein is also involved. But here, it induces the expression of other proteins different from PRs (Conrath et al., 2002). In addition to these pathways, research with new beneficial agents and different pathways are being described, especially since the ability of a PGPR to induce systemic resistance depends on the plant-beneficial bacteria–pathogen system. There is evidence of certain PGPR that are able to elicit systemic protection against *P. syringae* DC3000 in *A. thaliana* involving the SA-mediated pathway (Ramos Solano et al., 2008b).

SAR and ISR responses lead to plant protection against different pathogen species, but there are species which overlap. However, both responses can coexist in the same plant at the same time (van Wees et al., 2000). Thus, the use of PGPR or PGPR mixes able to trigger both responses at same time would result in an important advance in the improvement of pest defense systems.

The induction of defense metabolism, in fact, involves an induction of secondary metabolism developed by sessile organisms that are adapted to survive any changes of a biotic and abiotic nature. Therefore, some PGPR may trigger secondary metabolism against pathogens that at the same time may also be effective against biotic stress, such as saline conditions in soils, a frequent situation in agriculture. Furthermore, when a medicinal plant is used, phytopharmaceutical levels may also be increased or even new molecules may appear. This topic will be discussed in Section 8.4.

8.3 Application of PGPR for Agricultural Purposes

The use of PGPR in agriculture is one of the most interesting alternatives for improving sustainable agricultural practices, as well as for recovering degraded ecosystems (Vessey, 2003). PGPR can be used for a wide range of purposes that include

biofertilizers, biocontrol agents, induction of systemic resistance, and elicitors of secondary metabolic pathways that lead to products of nutritional and pharmacological interest and, therefore, of economic interest.

In view of the mechanisms described in Section 8.2, three case studies will be discussed: (1) suitability of the PGPR for nutrient-related traits; (2) alteration of plant metabolism mediated through plant hormonal balance; and (3) alteration of soil communities and their relation to biological effects. They highlight important aspects for successful application in agriculture.

Case study: A screening for PGPR to improve the growth of *Cistus ladanifer* (Gum Rockrose) seedlings for reforestation of degraded Mediterranean ecosystems was carried out by Ramos Solano et al. (2007). This screening for PGPR was carried out in the rhizosphere of wild populations of *C. ladanifer*, with the aim being to identify putative strains that are able to enhance the mycorrhization ability of *Cistus* with its spontaneous mycorrhizal fungus, *Amanita ponderosa*. The aim of identifying these strains was two-fold: one due to the great economic interest in the edible fruiting body of *Amanita* and, the second objective, helping the mycorrhization for soil recovery. Two hundred and seventy bacteria were isolated, purified, and grouped by morphological criteria. Fifty percent of the isolates were selected and tested for aminocyclopropanecarboxylic acid (ACC) degradation, auxin and siderophore production, and phosphate solubilization. Fifty eight percent of the isolates showed at least one of the evaluated activities, with phosphate solubilization and siderophore production being the most abundant traits. This is consistent with the chemical composition of the soil sampled, since usually the areas where *C. ladanifer* grows consist of a degraded soil or one that is in a regeneration stage following strong perturbation. A genetic analysis was performed with all strains that showed at least one positive trait. After PCR-RAPDs (randomly amplified polymorphic DNA) analysis, 11 groups appeared with 85% similarity, revealing the low diversity in the system. One strain of each group was tested in a biological assay, and those that enhanced *Cistus* growth were identified by 16S rDNA sequencing.

Although 7 of the 11 assayed strains were phosphate solubilizers and able to produce siderophores, only one was really effective in increasing all biometric parameters in *C. ladanifer* seedlings. This suggests that other mechanisms apart from nutrient mobilization might be involved in growth promotion by this strain. The lack of effect of the other six strains was probably due to the rich substrate used (peat) that diminishes the putative beneficial effect of the bacterium. Since this trait is not useful for this condition, genes are not expressed. However, the low diversity, together with the high redundancy detected by PCR-RAPDs and the predominance of strains able to mobilize nutrients in the rhizosphere of *Cistus*, reveals that the plant selects for bacteria that can help to supply scarce nutrients. These types of plant growth-promoting rhizobacteria (PGPR) strains should be successful in reforestation practices or in agricultural soils where phosphate or iron is present but not available for plants. This occurs under extreme conditions, where the PGPR represent a selective advantage for the plant.

The above case study shows that nutrient-related traits are not always useful for growth promotion (indirect mechanisms). Inoculation of nutrient-mobilizing bacteria will only result in positive results where nutrients are scarce. Therefore, bacterial strains showing nutrient-related traits should be used to increase productivity in areas with low yields due to lack of nutrients, not in rich cropping soils where chemical fertilizers are used and would result in "silencing" of the bacterial effect. In these latter soils, they should be used to lower the input of chemical fertilizers into the system.

However, the same PGPR strain may act by several mechanisms or may use different ones. The following case study illustrates another aspect of agriculture where plant nutrition cannot be further improved and the bacterial strain is able to improve yield under intensive greenhouse conditions. This strain is able to produce plant growth regulators using direct mechanisms.

Case study: The aim of this case study is to show how the inoculation of one PGPR strain is able to produce plant growth regulators (gibberellin and auxin types), using intensive culture, that improve productivity and protect the plant. It is within the aim of this study to show that inoculation of such biologic agents can be useful and feasible in current agricultural practices.

The effects of inoculation with a strain of *B. licheniformis* on the growth of pepper and tomato were investigated in three experiments. Before field trials, the survival of the strain against pesticides used in greenhouse production was tested to discard any possibility of failure due to pesticides; the bacterium was tolerant to most of them, leaving a possibility to be a part of integrated pest management (IPM). Of the three experiments, one was carried out under seedbed conditions and two under greenhouse production conditions. In the first experiment, the bacterium significantly increased the height of the plants and the leaf area in both species and in both cultivars. This is interesting for the production of plantlets with better adaptative capacities that will have better performance when transplanted to production greenhouses. Effects were more marked on pepper than on tomato, revealing certain specificity between the strain and the plant species. In the second experiment, seedlings growing in sand and in hydroponic culture were studied. The number and diameter of tomato fruits produced in sand and in hydroponic medium were increased significantly by PGPR inoculation, revealing the effects of gibberellins released by the PGPR. Given the physiological effects of this PGPR on flowering, it is interesting that flowering took place 15 days earlier in inoculated plants. This would result in the fruit reaching the market 2 weeks before the expected time. Hence, one can see the putative benefits for producers using these types of inoculants. In addition, PGPR-treated plants showed a lower incidence of disease than non-treated plants (no pesticides were used in either block), revealing a systemic induction of defensive metabolism by the PGPR. The effect could also be attributed to the simple colonization that could be impeding soil pathogen colonization (termed *niche exclusion*). In the third experiment, the total weight of pepper fruits harvested from PGPR-inoculated plants increased significantly as compared with non-inoculated controls. In light of the considerable colonization

and competitive ability of this PGPR strain and its effects on growth and plant physiology, it could be used as a biofertilizer or biocontrol agent without altering normal management in greenhouses. This would allow for lower chemical inputs for pathogen control.

However, the effects reported for greenhouse production have been validated only for those conditions per se (that is, extreme nutrient control and hydroponic or sand support). When this same bacterial strain is inoculated into soil containing its natural communities, the effect can be different. As a matter of fact, among the number of studies conducted on this topic, two will specifically be addressed in the third case study. But the general rule is that a strong alteration of native soil communities results in a lack of effect on plant growth, while a slight alteration of soil communities after PGPR inoculation is coupled with good results for plant growth.

Case study: This study is concerned with the influence of an indigenous European alder (*A. glutinosa* L. Gaertn) rhizobacterium (*B. pumilus*) on the growth of alder and its rhizosphere microbial community structure in two soils (Ramos et al., 2003). The aim of this study is to show that alteration of native communities of soils is negatively related to biological effects. European alder seedlings were inoculated with a suspension of the putative plant growth-promoting rhizobacteria (PGPR) *B. pumilus* (CECT 5105), or left non-inoculated (controls) in two different soils, and grown under controlled conditions. Soil A showed a coarse texture, was slightly acidic, and possessed a high nitrogen content, while soil B showed a fine texture, was basic in pH, and possessed a lower nitrogen content. The bacterium was isolated from soil A. At each sampling time, over an 8-week period, shoot and root systems of the plants were measured, determining shoot and root length and surface area; the number of nodules produced were counted. In addition, changes in the microbial rhizosphere structure were evaluated by the phospholipid fatty acid (PLFA) profile after extracting directly from the rhizosphere soil.

The increases detected in shoot surface were significant only in soil A, while the root system was affected in both soils, revealing the ability to produce auxin-like compounds of this strain that elicited better growth of the root system. However, while in soil A inoculation with *B. pumilus* caused a perturbation that subsequently disappeared, the rhizosphere community structure was seriously altered in soil B. This effect is based on the different profiles of phospholipids/fatty acids that indirectly reveal changes in the composition of microbiota. All biometric parameters were enhanced to a greater extent in soil A, in which the PGPR inoculum did not alter the existing rhizosphere communities, and nutrient availability was better. This is consistent with the previous hypothesis that release of auxin-type plant growth regulators by inoculated PGPR will result in better growth of the root system, which improves plant nutrient absorption potential. If this is coupled to enhanced nutrient availability, plants will show an increase in their growth parameters. Also, results were worst in soil B, in which case the PGPR strain had been isolated from soil A. In addition to the problems that inoculation caused in rhizosphere communities, there might be a nutrient dependence from the original soil that could condition the synthesis of growth regulators or any other factors that could affect growth.

As a concluding remark from this case study, it is clear that the influence of the soil cannot be discarded as a factor that has a negative influence on the beneficial effects of PGPR. Thus, it should be considered so as to increase success when releasing inoculants in soils. Although this case study was developed for a tree, not an agronomic or vegetable crop, this concluding remark applies to any crop species where the soil nature may condition the success of an added biofertilizer.

8.4 PGPR Affects Secondary Metabolism

Because plants have evolved secondary metabolism strategies to survive changing biotic and abiotic conditions encountered during their existence, this has also allowed them to colonize most habitats. Given the number of possibilities of changing conditions, both for biotic and abiotic factors, the array of secondary metabolites designed for each situation is enormous. Secondary metabolites can be studied from different points of view, such as the above examples for agriculture, the evaluation of their role in a plant's defense against pathogens, and their effects on human health. The following examples have been selected to illustrate each of them.

8.4.1 Lowering Chemical Inputs by Enhancing a Plant's Defensive Responses

When secondary metabolism is studied from the plant's defense point of view, the main goal is to meet requirements for sustainable development so as to achieve the highest yields. The mechanisms involved can be direct or indirect. If the bacterium used triggers the plant's defensive metabolism, it is a direct mechanism; if the bacterium is not able to trigger the plant's metabolism, but is able to interact with other microorganisms (biocontrol), it is an indirect mechanism. Moreover, they can happen simultaneously, achieving even better results than if they rely on a single microorganism, or rely on different ones that may show complementary beneficial traits, as for example, those related to nutrient mobilization.

The *case study* presented here pertains to rice: (*Oryza sativa* L.) production in Southern Spain (Seville) (unpublished data) and brings together two aspects of induction of secondary metabolism: (1) protection against pathogens and (2) protection against salinity inherent to rice cropping in this area.

The study was set up in Seville by the Guadalquivir River. Two PGPR strains were tested in experimental plots in the area, namely, *Chryseobacterium balustinum* and *P. fluorescens*. Both of them have demonstrated their ability to induce systemic resistance in *A. thaliana* (L.) Heynh. against *P. fluorescens* DC3000, *C. balustinum* being more effective (Ramos Solano et al., 2008), and has also shown biocontrol activity against *Rhizoctonia* (Doménech et al., 2006). *C. balustinum* has, in addition, demonstrated its ability to induce protection against saline conditions in *A. thaliana* (L.) Heynh. (Barriuso et al., 2008b). With these two candidates, five treatments were set up: each of the strains alone, the two combined, and two controls, one under

regular phytochemical treatments and the other without any addition of chemicals. Interestingly, the addition of bacterial treatments, once in the seed and then followed by several aerial inoculations during the growing season, resulted in a small increase in yield, but in addition, caused a large decrease in disease incidence under natural conditions. It is striking that the combination of the two strains achieved the best results. In light of these results, a combined strategy is going to be proposed for their use in integrated pest management. In this case, chemical pesticides can be applied if disease incidence runs out of control when the biological agent is used.

8.4.2 Increase in Secondary Metabolites of Pharmaceutical Interest

Special interest has been paid to plant species of medicinal interest due to their role in human health. Ever since plants have been used for healing, plant collectors have selected those with a better effect on health. These differences, in effect, are attributable to either higher levels of phytopharmaceuticals or differences in their relative contents, which most of the time are affected by environmental conditions when harvested from the field. A third possibility to explain this is that, for a known medicinal plant species, new molecules with pharmacological interest may appear when it is grown under a new set of environmental conditions or with a stress factor. Therefore, this would account for the improvement in its beneficial effects for human health.

The variability of secondary metabolism is a problem for the pharmaceutical industry since field production is uncertain and may condition availability of final products. Sometimes, the problem may be solved by chemical synthesis, but it is not always possible or at least it usually lacks economic feasibility. Another alternative is cell culture, but for some plant species, it is not feasible, or yields achieved are too low because secondary metabolism is not necessary under such controlled and undifferentiated conditions. Therefore, one of the main goals in industry now is to obtain reproducible extracts of plants grown in field production or, even more challenging, grown in plant cell cultures. For this purpose, the use of *elicitors* appears to be an encouraging alternative (Radman et al., 2003). In support of this last statement, a recent study by Poulev et al. (2003) has reported the potential of elicitation to discover new molecules with pharmacological interest. But this study not only reports the presence of new molecules but also the use of elicitors has been able to duplicate the presence of these molecules and to increase the concentration of known compounds. Hence, unraveling the nature of elicitors and the elicited pathways remains an exciting challenge for the pharmaceutical industry.

Among these putative elicitors, PGPR appear as good candidates for upregulating secondary metabolism. The following case study (Gutiérrez Mañero et al., 2003) illustrates how PGPR strains isolated from the rhizosphere of wild populations of *Digitalis* are able to enhance levels of cardenolides in high-yielding varieties of *Digitalis lanata* Ehrh., grown either in field production or as in vitro cell cultures.

Case study: The 480 isolates from a bacterial screening assay carried out in the rhizosphere of two *Digitalis* species in two physiological stages were characterized at the generic level. *Bacillus* was the dominant genus in all cases. Fifty percent of the *Bacillus* strains isolated from each species were analyzed by PCR-RAPDs. At 85% similarity, 12 groups separated out for *D. thapsi* L. and 18 for *D. parviflora* Jacq. One strain of each group was selected for biological assay on high-yield selected varieties of *D. lanata* Ehrh., kindly provided by Boehringer Mannheim (Spain). The evaluated parameters were growth promotion and cardenolide content in leaves. Inoculation was performed in the root system so as to obtain a systemic induction of secondary metabolism. Only 17 strains caused significant increases in at least one of the parameters evaluated. The most striking result was that some strains promoted growth and increased cardenolide content at the same time. This effect was detected in leaves, while inoculation was carried out in roots. Interestingly, these two parameters are not enhanced simultaneously under regular conditions of pot culture or in tissue cultures. This result shows that the biotic agent employed was able to upregulate the plant's metabolism. Moreover, it was striking that bacterial strains selected from wild species were able to upregulate secondary metabolism in selected varieties, especially for terpenes and cardenolides. The implication of this study is that identification of eliciting agents may now make it possible to enhance secondary metabolism in undifferentiated tissue cultures. This could be of major economic importance for the pharmaceutical industry because it would make possible either field production with higher yields or biotechnological production in vitro with good yields.

8.4.3 Modification of Secondary Metabolite Profiles of Pharmaceutical Interest

The effects of secondary metabolites on human health may vary depending on several factors. One of them is how they are delivered and incorporated into the human body, either in a pharmaceutical formulation, as food supplements, or in the diet. A pharmaceutical formulation will provide known concentrations of well-characterized and identified compounds, while a food supplement will provide an extract of the plant that possesses variable concentrations of known and unknown compounds. The most variable input is seen through the diet. Some edible plants, such as soybeans or berries, contain bioactive compounds that provide more benefits than is attributed to their simple nutritional value. Levels of bioactive compounds do change depending on environmental conditions; hence, the lack of an effect on health may be due to a lack of reproducibility of the bioactive content. This lack of reproducibility may be overcome by elicitation with biotic agents. However, the effect of elicitation needs to be evaluated because variation in relative concentrations of bioactive compounds may change their effects on human health when delivered in the diet. Besides changing the relative profiles, some interesting metabolites can be increased. This may be a very interesting tool for pharmaceutical companies as a means to prepare normalized extracts of food supplements.

*Case study: Biotechnological elicitation of isoflavones in Glycine max with PGPR. Diploma de estudios avanzados (DEA) Elena Algar Parejo. Universidad San Pablo CEU (unpublished results).*The rationale for this study was to evaluate the ability of PGPR to elicit soybean growth and secondary metabolism in terms of isoflavone production .

In a study on biotechnological elicitation of isoflavone production in soybeans (*Glycine max* (L.) Merr.) with PGPR by Elena Algar Parejo at the Universidad San Pablo CEU (unpublished results), a battery of nine PGPR with diverse beneficial capacities on different plant species were evaluated. None of them increased significantly the concentration of isoflavones according to the isoflavone standards that were evaluated (daidzein, daidzin, malonyl daidzin, genistein, genistin, malonyl genistin). However, when considering daidzein and genistein, five PGPR caused a decrease in their concentration, especially for genistein; one strain increased levels of both isoflavones; and two increased daidzein and decreased genistein levels. Decreases in levels of isoflavones may be coupled to increases in levels of protective compounds such as pterocarpanes. Although isoflavones have been traditionally related to defense against pathogens, it seems that pterocarpanes, a family of compounds derived from daidzein, are the compounds responsible for effective defense. Another putative reason for this decrease may be that isoflavones are released from the roots to the surrounding soil, creating an outbound gradient. The rationale for this is that they are involved in establishment of the symbiosis with nitrogen-fixing *Rhizobia* bacteria. Irrespective of the reasons underlying the different behaviors of each PGPR, the fact is that there is a difference depending on the strain and that it is a reproducible effect. It remains to be seen if these changes are relevant for human health through the diet. If this were the case, this opens a new window for the food supply industry.

8.5 Bacterial Cell Wall Lipopolysaccharides Are Able to Mimick PGPR-Mediated Induction of Defense Metabolism

Communication between plants and microorganisms is a well-known fact consistent with the many reports in the literature. Relations between plants and microorganisms may be beneficial or harmful. Irrespective of their nature, there is a common "language" used by both partners which is now starting to be elucidated. Some plant secondary metabolites such as isoflavones are understandable signals for specific microorganisms, namely, rhizobia. Some plant growth regulators are released by soil microorganisms that affect the plant's metabolites by targeting the plant's receptor (see above). But still, there are other molecules, mainly bacterial or fungal surface molecules, that are able to induce some receptors in plant tissues to start a particular signaling pathway. The term "elicitor" includes all of those molecules of different chemical nature, either constitutive of the microorganism or released, that are able to upregulate a plant's defense metabolic pathways (SAR and ISR pathways). This is especially relevant in plants with medicinal applications.

Biotic elicitors are classified into several groups that include proteins, polysaccharides, lipopolysaccharides, and volatile compounds. Polysaccharides are the most frequent elicitors described. Identification of these elicitors is essential for the practical application of defense responses both for agricultural and industrial purposes, since some of the defensive compounds are molecules with pharmacological activity. Furthermore, they can be used to elicit plant tissues in vitro, opening a promising window for biotechnological production of phytopharmaceuticals.

Case study: Ramos Solano et al. (2008b) have recently reported on systemic disease protection elicited by plant growth-promoting rhizobacteria (PGPR) strains in order to examine the relationship between metabolic responses, systemic disease protection, and biotic elicitors. They were able to demonstrate that a fraction of the bacterial cell wall is able to reproduce the defensive effect of the whole bacteria inoculated on plant roots.

The rationale of this study was first to evaluate the ability of three PGPR to elicit defensive metabolism in the model plant *A. thaliana* Col 0 against *P. syringae* pv. *tomato* DC3000, focusing on the putative induction pathway by biochemical and molecular markers. Second, it was carried out in order to demonstrate that bacterial cell wall surface molecules were able to reproduce the effect of the bacterial strain. The PGPR employed included *C. balustinum* (AUR9), *Azospirillum brasilensis*, and *P. fluorescens* (AUR6). All three strains decreased disease severity when applied to *A. thaliana* prior to pathogen challenge. At a biochemical level, each of the three strains induced ethylene (ET) biosynthesis when incubated with 1-amino-cyclopropane-1-carboxylic acid (ACC) as well as salicylic acid production in the plant. Plants treated with each of the three strains showed lower levels of salicylic acid after pathogen challenge compared to untreated controls, revealing a previous contact with an eliciting agent, as described for plants that have survived a pathogen attack (SAR). This effect was more marked in plants treated with *C. balustinum* AUR9, the strain most effective in decreasing disease severity. When this response was evaluated at the molecular level, the expression level of *PR1*, a transcriptional marker of the SA-dependent pathway, in *C. balustinum* AUR9-treated plants is four-fold that of controls, while the expression of *PDF1.2*, a transcriptional marker for the SA-independent pathway, is not induced. This suggests that this PGPR strain is inducing the plant defensive pathway that is dependent on SA and one that has traditionally been associated with pathogenic microorganisms. Once this systemic protective effect was demonstrated *in vivo*, inoculating the bacterial strain *C. balustinum* on roots and evaluating disease incidence on leaves, cell wall lipopolysaccharides were tested as putative bacterial elicitor molecules on axenic cultures of *A. thaliana* (L.) Heynh. Putative elicitors were dissolved in the culture media and were able to reproduce this systemic induction effect at low doses (5 $\mu g \cdot mL^{-1}$), but failed to reproduce the effect at higher doses (50 $\mu g \cdot mL^{-1}$). From these observations, we can hypothesize that certain PGPR strains are capable of stimulating different systemic responses in host plants. With *C. balustinum* AUR9, the SA-dependent pathway is stimulated first, as indicated by increases in SA levels and *PR1* expression, followed by induction of the SA-independent pathway, as indicated by the increases in ET concentrations. The effects on both pathways

combined, with respect to disease suppression, appear to be additive. Irrespective of the defensive pathway involved, the ability of LPS to elicit the defensive pathway has been demonstrated. This provides a challenging alternative for elicitation of secondary metabolism pathways in medicinal plants.

8.6 Quorum Sensing Is Involved in PGPR-Mediated Systemic Resistance Against Abiotic Stress

Quorum sensing (QS) is a widespread, rapid means for bacterial communities to coordinately change genome expression patterns in response to environmental cues and population density. The term quorum sensing was first used in a review by Fuqua and Winans (1994), which essentially concerned the minimum threshold level of individual cell masses required to initiate a concerted population response. Bacteria that use QS produce and secrete certain signaling compounds called *autoinducers*, or normally, *N-acyl-homoserine lactone* (*AHL*). The bacteria also have a receptor that can specifically detect the inducer. When the inducer binds to the receptor, it activates transcription of certain genes, including those for autoinducer synthesis. When only a few other bacteria of the same kind are in the vicinity, diffusion reduces the concentration of the inducer in the surrounding medium to almost zero. So, as a result, the bacteria produce only small amounts of the inducer. When a large number of bacteria of the same kind are in the vicinity, the inducer concentration crosses a threshold, whereupon greater amounts of the inducer are synthesized. This forms a positive feedback loop in which the receptor becomes fully activated (Whitehead et al., 2001).

Many gram-negative bacteria utilize AHL in order to coordinate expressions of virulence in response to the density of the surrounding bacterial population. Several bacterial phenotypes essential for the successful establishment of symbiotic, pathogenic, or commensal relationships with eukaryotic hosts utilize motility, exopolysaccharide production, biofilm formation, and toxin production to show regulation by QS (Gonzalez and Keshavan, 2006). It has been demonstrated that bacterial AHLs are released into the rhizosphere, where they reach biologically active concentrations (Barriuso et al., 2008d). This study shows how QS is involved in disease incidence, where several pathogenic microorganisms such as *Xanthomonas campestris*, *Erwinia carotovora*, *Pseudomonas corrugata*, or *Burkholderia* sp. strains are the causal agents (Ahmad et al., 2008).

Interestingly, the production of quorum-sensing interfering (QSI) compounds by eukaryotic microorganisms has aroused immense interest among researchers, especially since such compounds can influence the bacterial signaling network positively or negatively. Hence, strategies addressing disruption of QS among bacterial strains appear to be an encouraging and novel strategy for plant health protection, especially when supported by the fact that some plant species do release AHL-like compounds to modulate bacterial communication in the rhizosphere (Teplistsky et al., 2000). On the contrary, synthesis of structural homologues to various QS signal molecules

has resulted in the development of additional QSI compounds that could be used to control pathogenic bacteria. Furthermore, the creation of transgenic plants that express bacterial QS genes is yet another strategy that could be utilized to interfere with bacterial behavior (Fray, 2002).

Case study: In a study on transgenic tomato plants that alter *quorum sensing* in plant growth-promoting rhizobacteria (PGPR), Barriuso et al. (2008d) showed that growth promotion and salt resistance are mediated by QS. This, then, extends the roles of QS and reinforces the concept of disruption of QS to control plant pathogen attack or to induce protection effects against different kinds of stresses. Details on this study are provided in the following account.

Two gram-negative plant growth-promoting rhizobacteria (PGPR), designated as M12 and M14, affiliated with *Burkholderia graminis*, could produce a variety of *N*-acyl-homoserine lactone (AHL) signaling molecules. The involvement of these molecules in plant growth promotion and induction of protection against salt stress was examined. AHL production was evaluated in vitro by thin-layer chromatography and bioindicator detection as well as by LC-MS/MS. In situ production of AHLs in the rhizosphere of *A. thaliana* (L.) Heynh. plants was detected by GFP (green fluorescent protein) biosensor constructs and confocal laser scanning microscopy. To determine if plant growth promotion and protection against salt stress were mediated by quorum sensing (QS), these PGPR were assayed on wild-type (wt) tomato plants as well as their corresponding transgenics expressing YenI (short-chain AHL producers) and LasI (long-chain AHL producers). In wt tomato plants, only M12 promoted plant growth, and this effect disappeared in both transgenic lines. In contrast, the strain M14 did not promote growth in wt tomatoes, but did in LasI. Resistance to salt stress was induced by strain M14 in wt tomato, but this effect disappeared in both transgenic lines. Strain M12, however, did not induce salt resistance in wt tomato, whereas it did in LasI tomato plants. These results reveal that QS AHL signal molecules mediate the ability of both PGPR strains, M12 and M14, to promote plant growth and to induce protection against salt stress.

8.7 Metagenomics: The Rhizosphere as a Source of Genes with Biotechnological Applications

A relative new perspective in the biotechnology of the rhizosphere is the *metagenomic approach* (Rolf, 2005). This approach is defined as the field of molecular biology whose purpose is to study the genome of entire communities of microorganisms instead of individual species. Also known as "communities genomics", this discipline examines the genetic material recovered from environmental samples derived from entire communities of microorganisms. Classical microbiology and genetics, in trying to isolate genes with new and valuable functions, basically are based on the isolation of single microorganisms and the sequencing of their genomes. Metagenomics allows the study of non-culturable, or difficult-to-culture, microorganisms. This approach gives a new estimation of microbial communities independent of their culture in the laboratory (Rolf, 2005).

Rhizosphere metagenomics examines the total DNA extraction from the rhizosphere, the preparation of a clonal library with this DNA, and the screening of the clones to select the genes of interest among the vast genetic reservoir derived from soil microbial communities.

The metagenomic approach has already been used for the identification of new biomolecules based on the finding of genes codifying proteins with new activities. This strategy has been validated by the isolation of novel genes that encode degradative enzymes (Henne et al., 1999), antibiotic resistance (Riesenfeld et al., 2004), and antibiotic production (Wang et al., 2000). However, association of a certain gene with its activity is quite difficult, and sometimes, the limiting factor is that the genes in question may not be expressed at detectable levels. Despite the tedious and long process of gene isolation and identification, rhizosphere metagenomics appears to be a very promising field to find new genes with novel activities and high biotechnological value, but also implicit are its own technical limitations that have to be overcome to assure new technical advances in the future.

8.8 Metabolic Engineering

The relevance of this perspective is fully described in Chapters 2 and 12. Nevertheless, a short discussion is needed here to examine the role of bacterial elicitors in metabolic engineering. Despite the efforts devoted to increase and diversify bioactive compounds in plants, it is still a challenge as to how to increase their content in vivo and, as mentioned before, how to obtain reproducibility of bioactives under field production conditions. These efforts rely on transgenic and non-transgenic approaches which involve complex regulation mechanisms that are required for increasing the levels of functional metabolites in plants. Bacterial elicitors may be used to determine the key genes limiting a metabolic pathway once the limiting step is identified. Transgenic approaches may allow us to overcome the low levels of target compounds produced. Finally, and this is a very attractive and encouraging challenge, upon elicitation, new molecules may appear after the activation of a given metabolic pathway.

8.9 Future Perspectives

The future of PGPR research should involve all aspects that are highlighted in this chapter. One of them is the selection of PGPR or PGPR mixes to help solve current agricultural problems, including limiting the use of highly contaminating pesticides and fertilizers and allowing for crop cultivation in low-fertility soils.

Although sustainable agriculture is directly related to human health, the use of PGPR to enhance levels of secondary metabolites that directly affect health through the diet may lead to the creation of *functional foods*, that is, foods having a beneficial effect on human health, that are superior to the benefits ascribed to their simple nutritional value.

Finally, identification of specific elicitors of bacterial origin may be used, either in agriculture, in the production of phytopharmaceuticals in vitro under controlled conditions, or at a physiological/molecular level, to study plant metabolism that allows us to unravel the limiting steps of metabolic pathways.

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