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# **Bacillus-Based Biological Control of Plant Diseases**

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## **1. Introduction**

Plant diseases cause considerable losses in crop production and storage. Nowadays, growers still rely heavily on chemical pesticides to prevent, or control these diseases. However, the high effectiveness and ease of utilization of these chemicals can result in environmental contamination and the presence of pesticide residues on food, in addition to social and economic problems. Consequently, there is an increasing demand from consumers and officials to reduce the use of chemical pesticides. In this context, biological control through the use of natural antagonistic microorganisms has emerged as a promising alternative. Indeed, these biopesticides present many advantages in term of sustainability, mode of action and toxicity compared to chemical pesticides. Here, we focus in details on the versatile utilization of *Bacillus* based products as biopesticides. More precisely, a special emphasis is given to the three main specific mechanisms involved in biocontrol of plant diseases by this bacterial genus: competition for ecological niche/substrate in the rhizosphere, production of inhibitory chemicals and induction of so-called systemic resistance in host plants. Beside this, strategies for enhancing the efficacy of *Bacillus*-based biopesticides are also discussed.

## **2. Potential for microbial biocontrol agents in agriculture**

### **2.1 Interest in the development of biopesticides**

As all living organisms, plants must face infections and diseases following the attacks of a mass of plant pathogens and pests from animal, microbial or viral origin. These diseases can be minor causing solely a reduction of plant-growth capacities or can be at the origin of much more severe damage leading to plant death in the worst case. Plant diseases are responsible for the loss of at least 10% of global food production, representing a threat to food security (Strange & Scott, 2005). Agrios (2004) estimated that annual losses caused by disease cost US\$ 220 billion. Worldwide, plant diseases were responsible for severe famines in the past (Agrios, 2004). For example, potato blight caused by the plant pathogenic oomycete *Phytophthora infestans* on potato cultures caused more than one million deaths in Ireland during the “the great famine” between 1845 and 1849 (O'Neill, 2009).

To prevent or control these diseases, producers have become increasingly dependent on agrochemicals, especially over the past few decades, as agricultural production has intensified. However, despite the great effectiveness and ease of utilization of these products, their use or misuse has caused many problems including significant pollution of soils and ground water reservoirs, accumulation of undesirable chemical residues in the food chain, emergence of fungicide-resistant strains of pathogens, not to mention health concerns for growers (Fig. 1). According to the Stockholm convention on persistent organic pollutants, 10 of the 12 most dangerous and persistent organic chemicals are pesticides (Gilden et al., 2010). An example is the synthetic pesticide dichlorodiphenyltrichloroethane, well known as DDT, which was extensively used in agriculture between 1950 and 1980 and was found genotoxic in human and responsible for endocrine disorders (Cohn et al., 2007). Consequently, there is nowadays an increasing demand from consumers and authorities for more safe, rational, sustainable and eco-friendly strategies. This has resulted not only in stricter regulations concerning pesticide use, commercialization and production but also in the development of alternative strategies including genetic adaptation of crops, modification of cultural practices and use of biopesticides.

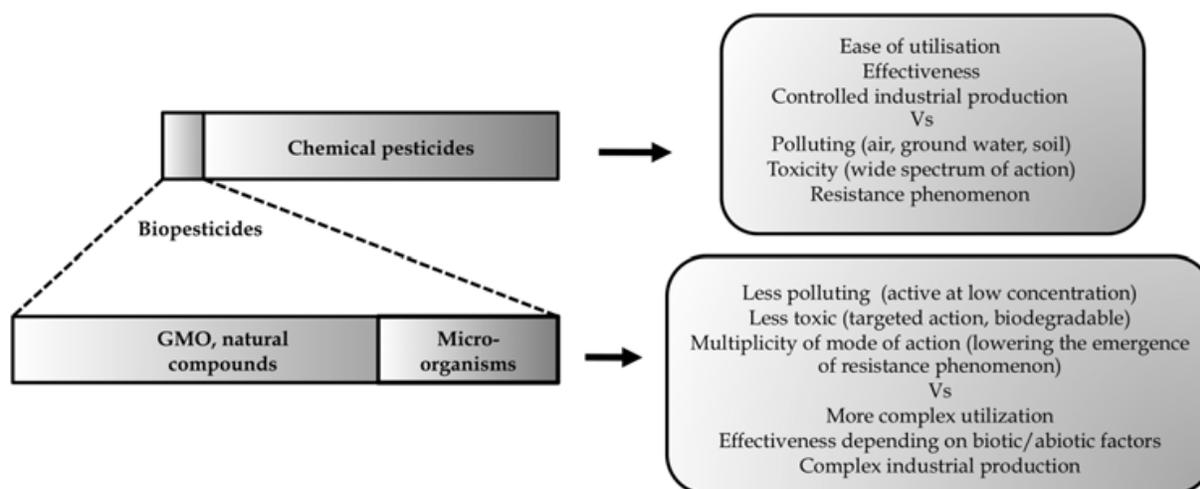


Fig. 1. Market share and (dis)advantages of microbial biopesticides versus chemical phytosanitary products.

## 2.2 Advantages and marketshare of biopesticides

Biopesticides, which are used to suppress pathogen populations, are living organisms or natural products derived from these organisms. They can be divided into four main groups: microorganisms (microbial pesticides), other organisms (nematodes, insects...) used to control pests, natural substances that are derived from living organisms (biochemical pesticides) and plant-incorporated protectants (genetically modified plants) (EPA, 2011; Thakore, 2006). Biopesticides show several advantages when compared to chemical products. They decompose more quickly in the environment and are generally less toxic towards non-target species (Thakore, 2006). Additionally, their modes of actions are usually distinct from those of conventional pesticides. This implies that they can often help suppress resistant pathogens and that they can be applied in alternation with other pesticides to avoid resistance development (see below and Fig. 1).

Among biopesticides, microorganism-based products represent about 30% of total sales and have a variety of applications. They are used in field crops and greenhouses to reduce

diseases on various cereals, legumes, fruits, flowers and ornamental plants caused either by soilborne, foliar or post-harvest pathogens. These plant protective microorganisms, mainly fungi and bacteria, are often isolated from suppressive environments. In other words, these beneficial microorganisms are generally obtained from aerial or underground parts of plants that are naturally less or not at all affected by a pathogen that devastates a neighboring group of the same plant species (Cook & Baker, 1983; Ryan et al., 2009). One of the advantages of microbial biopesticides compared to most other phytosanitary products is the multiplicity of their ways of actions globally based on competition for nutrients and space, direct antagonism of plant pathogen growth and host plant immunization (see below). Compared to GMOs, microbial pesticides benefit from a better consumer acceptance. In Europe, there are also several legal barriers against GMOs. In comparison with natural extracts, microbial pesticides often retain the advantage of having a persistent activity through time. Indeed, microbial agents can establish themselves in the phytosphere and produce continuously bioactive compounds *in situ*. Moreover, as these active molecules are produced in direct contact or very close to the target organisms, only limited quantities are needed for efficacy.

In addition to their potential to directly reduce the incidence of diseases, some microbial products also have other positive effects on crops such as promoting plant growth and nutrition (biofertilizers and phyto-stimulators) and/or facilitating interaction between the host plant and other beneficial organisms (Antoun & Prevost, 2006). A large amount of nutrients present in the soil are in an insoluble form that is unavailable for the crops (Francis et al., 2010). Biofertilizers act through the direct improvement of plant nutrition either by solubilizing these nutrients or by fixing atmospheric N<sub>2</sub>. In the case of solubilization, several mechanisms may be involved depending on the nature of the nutrient. For example, phosphate can be released from insoluble organic forms by several microbial enzymes like phytases or non-specific phosphatases, while inorganic phosphorus stocks are solubilized through the production of organic acids by the beneficial bacteria. Phyto-stimulation is the direct promotion of plant growth through the modulation of the plant's hormonal balance. Several microorganisms are capable to produce and excrete a variety of plant hormone-like compounds including auxin, gibberellins, cytokinins etc. Some microbial agents produce enzymes that degrade a precursor of ethylene thus limiting the levels of this hormone in the plant thereby increasing plant growth especially under stress conditions (Francis et al., 2010; Lugtenberg & Kamilova, 2009). Both biofertilization and phyto-stimulation are important phenomena in the context of the constant need to produce more food on fewer surfaces with the simultaneous wish to reduce reliance on chemical fertilizers. Moreover, a microorganism that possesses a combination of these growth-promoting activities and biocontrol potential offers the advantage to supply the crop in one application with a biopesticide and a biofertilizer. In addition, better nutrition of the plant often enhances its overall resistance against pathogens and other stress factors (Bent, 2006).

Despite providing such advantages, biopesticides take up only a small share of the pesticide market, 2.5% in 2005, which still represents an important business as global pesticide sales in 2005 reached 26.7 billion dollars. Biopesticides have gained more and more interest over the years with a market share in 2000 of only 0.2% and an expected 15% annual market growth. Moreover, conventional pesticides have been slowly losing ground since 2000, with an expected decline rate of 1.5% per year (Thakore, 2006).

### 2.3 Biopesticides and integrated pest management

Biopesticides thus play an important role and are legally accepted for use in integrated pest management and organic agriculture. According to the US Environmental Protection Agency (EPA), Integrated Pest Management (IPM) is an effective and environmentally sensitive approach that relies on a combination of common-sense practices (EPA, 2011). IPM programs use current, comprehensive information on the life cycles of pests and on their interaction with the environment. This information, in combination with available pest control methods, is used to manage pest damage by the most economical means, and with the least possible hazard to people, property, and the environment. IPM may involve a judicious use of pesticides by contrast with organic food production that applies many of the same concepts as IPM but limits the use of pesticides to those that are produced from natural sources, as opposed to chemicals.

An example of integration of alternative/biological methods in IPM is given here for the control of lily diseases and pests. This program was developed in a company specialized in the cultivation of lily, located in Holambra, SP, Brazil, with a history of intensive use of fungicides, insecticides and miticides. Phytosanitary problems in lily culture of high value, limit its cultivation. Diseases may originate from several agents such as the fungi/oomycetes *Botrytis elliptica*, *Phytophthora*, *Fusarium*, *Sclerotinia*, *Penicillium*, *Rhizoctonia* and *Pythium* or pests such as aphids, fungus gnats, leaf miners, thrips and caterpillars. To solve these problems, in 2000, over 30 different chemical pesticides had to be used routinely at a cost of US\$ 10.00/m<sup>2</sup>/year in a cultivated area of 13,500 m<sup>2</sup>. For these products to keep working properly, growers needed to use increasingly higher doses and more toxic products, but losses due to pests and diseases kept increasing. Facing such a situation, the decision was made to change the production system. To achieve integrated control of cultural problems, the use of chemical pesticides was gradually replaced by the integration of biocompatible methods to control pests and diseases like introducing a diversity of microorganisms for biocontrol. Along with this substitution of chemical pesticides, an adaptation of fertilization procedures was needed to improve the survival of the biocontrol agents. The first step was to stop using the most toxic pesticides which took about two years. One additional year was required to successfully replace the use of chemical pesticides of less toxic levels. In general, the current production is based on the treatment of a steam-disinfested substrate with aerobic compost tea and beneficial microorganisms such as *Trichoderma*, *Metarhizium*, *Beauveria* and *Bacillus*. *Clonostachys rosea* and *Trichoderma* sp. are sprayed weekly to control *Botrytis* and other pathogens. When necessary, neem oil, propolis, phosphite and others alternative products are used. Associated with these products and with balanced fertilization, a sanitation program is maintained in all the greenhouses with the elimination of diseased plants or plant's parts. Also, traps and monitors for controlling the relative humidity in greenhouses are used. Currently, no chemical pesticides are used, except for bulbs, which are treated with imidaclopride before planting to control aphids, in order to comply with phytosanitary standards for exportation. The success is due, not only to the substitution of chemical pesticides by biopesticides and biocompatible products, but also by reconsidering the entire production system. The acreage today is 27,500 m<sup>2</sup> with an approximate cost to control disease and pest problems of US\$ 3.00/m<sup>2</sup>/year (Wit et al., 2009). The same strategy is used for the control of disease on *Spathiphyllum*, avoiding any chemical pesticide input and involving *Bacillus subtilis* for the control of *Cylindrocladium spathiphylli* (Wit et al., 2009).

### 3. Interest in *Bacilli* as biopesticides

Bacterial products represent the majority of the microorganism-based biopesticides but fungal biocontrol agents were also developed as efficient products (recently reviewed by Shores et al., 2010). Among the bacterial biocontrol agents, *Bacillus thuringiensis* accounts for more than 70% of total sales. This bacterium is essentially used for insect pest control and is the origin of the gene used in insect resistant "Bt GMO crops". Other isolates from several bacterial genera have been used successfully for crop protection and numerous products listed in Table 1 are currently commercialized in the world for the control of important plant diseases (Table 1). As illustrated in this table, about half of the commercially available bacterial biocontrol agents are *Bacillus*-based products but strains of the other genera, including *Streptomyces* and *Pseudomonas*, were also marketed for biocontrol in the recent years.

The *Bacillus* genus encompasses a large genetic biodiversity. *Bacilli* are present in an extremely large palette of environments ranging from sea water to soil, and are even found in extreme environments like hot springs (Hoch et al., 1993). This bacterium could be one of the major sources of potential microbial biopesticides because it retains several valuable traits (Ongena & Jacques, 2008). Firstly, *Bacilli*, such as *B. subtilis*, are well-studied organisms which facilitates their rational use. Secondly, the US Food and Drug Administration (USFDA) has granted the "generally regarded as safe" (GRAS) status to *Bacillus subtilis* which is thus recognized non-pathogenic (Harwood & Wipat, 1996). This is of course essential regarding its application as a biopesticide. Thirdly, *Bacilli* have the capacity to produce spores (Piggot & Hilbert, 2004) which are extremely resistant dormancy forms capable to withstand high temperatures, unfavorable pH, lack of nutrients or water, etc. They are produced by the bacteria when environmental conditions are unfavorable which probably helps these microorganisms to survive in the phytosphere. The phenomenon can also be exploited in industrial production as sporulation can be induced at the end of cultures (Monteiro et al., 2005). This greatly facilitates post-culture conditioning as bacterial suspensions can be converted to easy to handle powder formulations without the impressive bacterial mortality observed with non-sporulating bacteria (Lolloo et al., 2010). Shelf life of biopesticides based on sporulated bacteria is generally longer and require less storage precaution compared to other products containing living organisms. *Bacilli* are also relatively easy to produce industrially as they are not particularly exigent regarding nutritional sources. Beside its spore forming ability, *B. subtilis* possess several characteristics that enhance its survival in the rhizosphere and thus its effectiveness as a biopesticide (Losick & Kolter, 2008; Rosas-Garcia, 2009). This bacterium known to live in aerobic environments can also behave as facultative anaerobe surviving and evolving under low oxygen concentration (Nakano & Hulett, 1997). This is a real advantage in the rhizosphere as oxygen availability may fluctuate during time and is generally low. Additionally, *B. subtilis* is a motile bacterium that readily moves towards and on the root surface which facilitates colonization of new ecological niches. Another reason for the high interest in *Bacilli* is the diversity of their modes of action. They can display almost all the mechanisms of biocontrol and bio-stimulation/fertilization mentioned here below and above. Moreover, one strain may often acts through several mechanisms. This enables these bacteria to be effective in many conditions (variety of pathogens, plants, environmental conditions) as one mechanism may act instead of another.

| Product              | Bioagent / mode of action  | Diseases / target pathogens   | Crop  | Company                              | Registered and commercialized                  |
|----------------------|--|---|---|--------------------------------------|--|
| <i>Bacillus</i> spp. |  |   |   |                                      |  |
| Avogreen®            | <i>B. subtilis</i> / antibiosis  | <i>Colletotrichum gloeosporioides</i> and <i>Cercospora</i> spot  | Avocado   | Ocean Agriculture                    | South Africa                                   |
| Bacillus SPP®        | <i>Bacillus</i> spp./ antibiosis   | <i>Pseudomonas syringae</i> pv. <i>syringae</i> , <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> and <i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>   | Several crops   | Bio Insumos Nativa Ltda., Chile      | Chile  |
| Ballad®              | <i>B. pumilus</i> / antibiosis, competition, growth promotion and resistance induction | Root rot ( <i>Rhizoctonia oryzae</i> ), rust ( <i>Puccinia</i> spp., <i>Uromyces betae</i> , <i>Puccinia sorghi</i> ), rice blast ( <i>Pyricularia oryzae</i> ), powdery mildew ( <i>Peronospora manshurica</i> , <i>Erysiphe graminis</i> , <i>Erysiphe betae</i> , <i>Erysiphe polygoni</i> ), leaf spot ( <i>Cercospora</i> , <i>Cercospora beticola</i> ), <i>Entyloma</i> , <i>Dreschlera</i> , <i>Exserohilum turcicum</i> , <i>Helminthosporium</i> , <i>Bipolaris maydis</i> , <i>Cochliobolus heterostrophus</i> , <i>Cochliobolus</i> , <i>Ceratobasidium</i> , <i>Ramularia</i> ), bacterial spot ( <i>Xanthomonas</i> spp.), Asian soybean rust ( <i>Phakopsora pachyrhizi</i> ), brown spot ( <i>Septoria glycines</i> ), white mold ( <i>Sclerotinia sclerotiorum</i> ) | Cereals, oil plants, sugar beet                           | AgraQuest Inc., USA                  | USA  |
| Bio safe®            | <i>B. subtilis</i> / antibiosis  | Foliar blight   | Soybean, bean, cotton                                     | Lab. Biocontrole Farroupilha, Brazil | Brazil (not sold, only for use in the company) |
| Biosubtilin          | <i>B. subtilis</i> / antibiosis, competition   | <i>Fusarium</i> , <i>Verticillium</i> , <i>Pythium</i> , <i>Cercospora</i> , <i>Colletotrichum</i> , <i>Alternaria</i> , <i>Ascochyta</i> , <i>Macrophomina</i> , <i>Myrothecium</i> , <i>Ramularia</i> , <i>Xanthomonas</i> and <i>Erysiphe polygoni</i>   | Cotton, cereals, ornamental plants and vegetable crops    | Biotech International Ltd.           | India  |
| Botrybel             | <i>B. velezensis</i>   | <i>Botrytis cinerea</i>   | Tomato, lettuce, pepper, grape, strawberry and vegetables | Agricaldes, Spain                    | Spain  |
| Cease®               | <i>B. subtilis</i>   | Soilborne pathogens ( <i>Rhizoctonia</i> , <i>Pythium</i> , <i>Fusarium</i> , <i>Phytophthora</i> ) and foliar pathogens ( <i>Botrytis</i> , <i>Erwinia</i> , <i>Xanthomonas</i> )  | Several crops   | BioWorks Inc., USA                   | USA, Mexico                                    |

| Product                             | Bioagent / mode of action  | Diseases / target pathogens   | Crop  | Company   | Registered and commercialized |
|-------------------------------------|--|---|---|---|-------------------------------|
| Companion®                          | <i>B. subtilis</i> / antibiosis, competition, growth promotion, resistance induction | Root rots ( <i>Aspergillus</i> , <i>Golovinomyces cichoracearum</i> , <i>Fusarium oxysporum</i> , <i>Fusarium nivale</i> , <i>Magnaporthe poae</i> , <i>Phytophthora</i> , <i>Pythium</i> , <i>Rhizoctonia solani</i> , <i>Sclerospora graminicola</i> , <i>Sclerotinia minor</i> ), leaf spot ( <i>Alternaria</i> , <i>Botrytis cinerea</i> , <i>Colletotrichum orbicular</i> , <i>Colletotrichum</i> , <i>Didymella bryoniae</i> , <i>Erwinia carotovora</i> , <i>Erwinia tracheiphila</i> , <i>Plasmidiophora brassicae</i> , <i>Podosphaera xanthi</i> , <i>Pseudomonas syringae</i> , <i>Xanthomonas campestris</i> )  | Cotton, bean, pea, soybean, peanut, corn, and others        | Growth Products Ltd., USA                               | USA                           |
| EcoGuard™ Biofungicide              | <i>B. licheniformis</i> / antibiosis and enzymes                                     | Antracnose ( <i>Colletotrichum graminicola</i> ) and dollar spot ( <i>Sclerotinia homeocarpa</i> )  | Golf courses, sports turf, lawns, turf farms and arboretums | Novozymes A/S, Denmark. Novozymes Biologicals, USA      | USA                           |
| Ecoshot                             | <i>B. subtilis</i>   | Gray mold ( <i>B. cinerea</i> )   | Grape, citrus, vegetables, legumes and others               | Kumiai Chemical Industry Japan                          | Japan                         |
| FZB24®WG, li and TB                 | <i>B. subtilis</i>   | Root rot and wilts ( <i>Alternaria</i> , <i>B. cinerea</i> , <i>Curvularia radicola</i> , <i>Curvularia inequalis</i> , <i>Corynebacterium michiganense</i> , <i>E. carotovora</i> , <i>Fusarium avenaceum</i> , <i>Fusarium culmorum</i> , <i>F.oxysporum</i> f. sp. cucumerinum, <i>F.oxysporum</i> f. sp. dianthi, <i>F.oxysporum</i> f. sp. gerberae, <i>F.oxysporum</i> f. sp. gladioli, <i>F.oxysporum</i> f. sp. lycopersici, <i>F.oxysporum</i> f. sp. narcissi, <i>Gaeumannomyces graminis</i> , <i>Gerlachia niveale</i> , <i>Phoma chrysanthemi</i> , <i>Phomopsis sclerotioides</i> , <i>Pyrenochaeta lycopersici</i> , <i>P.ultimum</i> , <i>R. solani</i> , <i>S. sclerotiorum</i> , <i>Stromatinia freesia</i> , <i>Verticillium</i> spp.) | Several crops   | ABiTEP GmbH, Germany                                    | Germany                       |
| HiStick N/T® / Subtilex® / Pro-Mix® | <i>B. subtilis</i>   | Root rot and seed treatments ( <i>Fusarium</i> , <i>Rhizoctonia solani</i> , <i>Aspergillus</i> , <i>Pythium</i> and <i>Alternaria</i> )  | Soybean, ornamental plants and other crops                  | Becker Underwood, USA Premier Horticulture Inc., Canada | USA, Canada                   |
| Kodiak®                             | <i>B. subtilis</i> / antibiosis, competition, growth promotion, resistance induction | Soilborne diseases ( <i>Rhizoctonia</i> and <i>Fusarium</i> )   | Cotton  | Gustafson Inc., USA                                     | USA                           |

| Product                              | Bioagent / mode of action       | Diseases / target pathogens   | Crop  | Company              | Registered and commercialized   |
|--------------------------------------|---------------------------------|---|---|----------------------|---|
| Rhapsody®                            | <i>B. subtilis</i>              | Anthraco nose ( <i>Colletotrichum</i> spp.), bacterial leaf spot ( <i>Erwinia</i> , <i>Pseudomonas</i> , <i>Xanthomonas</i> ), leaf spot ( <i>Cercospora</i> , <i>Entomosporium</i> , <i>Helminthosporium</i> , <i>Myrothecium</i> , <i>Septoria</i> , <i>Diplocarpon rosea</i> ), gray mold ( <i>B. cinerea</i> ), downy mildew ( <i>Peronospora</i> spp.), early blight ( <i>Alternaria</i> ), powdery mildew ( <i>Erysiphe</i> , <i>Oidium</i> , <i>Podosphaera</i> , <i>Sphaerotheca</i> ), rust ( <i>Puccinia</i> ), scab ( <i>V. inaequalis</i> ), root rot ( <i>R. solani</i> , <i>Pythium</i> , <i>Fusarium</i> , <i>Phytophthora</i> ), dollar spot ( <i>Sclerotinia homeocarpa</i> ), rice blast ( <i>Pyricularia grisea</i> ), soilborne diseases ( <i>Rhizoctonia</i> , <i>Pythium</i> , <i>Fusarium</i> and <i>Phytophthora</i> )  | Turf, forest, ornamental plants   | AgraQuest Inc., USA  | USA, Canada   |
| Rhizo Plus®                          | <i>B. subtilis</i> FZB24        | Soilborne pathogens   | Gardening (Several crops)   | ABiTEP GmbH, Germany | Germany   |
| RhizoVital®42 li and RhizoVital 42TB | <i>B. amylolique-faciens</i>    | Soilborne pathogens   | Potato, corn, strawberry, tomato, cucumber, ornamental plants   | ABiTEP GmbH, Germany | Germany   |
| Serenade®                            | <i>B. subtilis</i> / antibiosis | Gray mold ( <i>B. cinerea</i> ), Botrytis ( <i>B. cinerea</i> ), black Sigatoka ( <i>Mycosphaeraella fijiensis</i> ), early blight ( <i>Alternaria solani</i> ), late blight ( <i>Phytophthora infestans</i> ), powdery mildew ( <i>Leveillula taurica</i> , <i>Oidiopsis taurica</i> , <i>Erysiphe chichoracearum</i> , <i>Erysiphe</i> spp., <i>Sphaerotheca macularis</i> , <i>Sphaerotheca</i> spp., <i>Podosphaera clandestina</i> , <i>Podosphaera leucotricha</i> , <i>Uncinula necator</i> ), downy mildew ( <i>Bremia lactucae</i> , <i>Peronospora</i> spp.), early leaf spot ( <i>Cercospora</i> spp.), Botrytis neck rot ( <i>Botrytis</i> spp.), scab ( <i>Venturia</i> spp.), leaf-drop ( <i>Sclerotinia</i> spp.), bacterial spot ( <i>Xanthomonas</i> spp.), walnut blight ( <i>Xanthomonas campestris</i> ), fire blight ( <i>Erwinia amylovora</i> ), anthracnose ( <i>Colletotrichum</i> ), white mold ( <i>Sclerotinia sclerotiorum</i> ) | Grape, apple, pear, banana, cherry, walnut, peanut, hop, leafy vegetables, tomato, peppers, cucurbits, mango, bean, onion, garlic, potato, broccoli, carrot | AgraQuest Inc., USA  | Chile, USA, New Zealand, Mexico, Japan, Israel, Costa Rica, Philippines, Guatemala, Honduras, Argentina, Italy, France, Turkey, Switzerland, Korea, Ecuador, Peru, and others |

| Product                             | Bioagent / mode of action   | Diseases / target pathogens   | Crop  | Company                                     | Registered and commercialized           |
|-------------------------------------|---|---|---|---|---|
| Sonata®                             | <i>B. pumilus</i>   | Powdery mildew ( <i>Oidiopsis taurica</i> , <i>Erisiphe</i> spp., <i>Erysiphe cichoracearum</i> , <i>Uncinula necator</i> , <i>Sphaerotheca</i> spp., <i>Sphaerotheca macularis</i> , <i>Podosphaera leucotrica</i> ), early blight ( <i>Alternaria solani</i> ), late blight ( <i>Phytophthora infestans</i> ), downy mildew ( <i>Peronosporaspa</i> , <i>Pseudoperonospora</i> spp., <i>Bremia lactucae</i> ) | Tomato, potato, grape, strawberry, cucurbits, peppers, apple, pear  | AgraQuest Inc., USA                         | USA, Mexico, Peru, Switzerland, Germany |
| Sublic®                             | <i>Bacillus</i> sp.   | Damping-off, root rot, and wilt ( <i>Botrytis</i> , <i>Rhizoctonia</i> , <i>Colletotrichum</i> , <i>Sclerotinia</i> , <i>Macrophomina</i> , <i>Phomopsis</i> and <i>Pythium</i> )   | Several crops   | ELEP Biotechnologies Italy                  | Italy                                   |
| Yield Shield®                       | <i>B. pumilus</i>   | Root rot ( <i>R. solani</i> and <i>Fusarium</i> )   | Soybean   | Bayer CropScience, USA                      | USA                                     |
| <b><i>Streptomyces</i> spp.</b>     |   |   |   |   |   |
| Actinovate® SP                      | <i>S. lydicus</i> / antibiosis, enzymes, competition, growth promotion          | Damping-off and root rot ( <i>Pythium</i> , <i>Rhizoctonia</i> , <i>Fusarium</i> , <i>Phytophthora</i> , <i>Verticillium</i> ), powdery mildew ( <i>Erysiphe</i> , <i>Oidium</i> , <i>Podosphaera</i> , <i>Sphaerotheca</i> ), downy mildew ( <i>Pseudoperonospora</i> , <i>Peronospora</i> ), gray mold ( <i>B. cinerea</i> ) and alternaria blight ( <i>Alternaria</i> spp.)                                  | Ornamental plants, vegetables, turf, forest species                 | Natural Industries Inc., USA                | USA                                     |
| Mycostop®                           | <i>S. griseoviridis</i> / antibiosis, competition, parasitism, growth promotion | Root rot, damping-off, and wilt caused by <i>Fusarium</i> , <i>Alternaria brassicola</i> , <i>Phomopsis</i> , <i>Botrytis</i> , <i>Pythium</i> , <i>Phytophthora</i> and <i>Rhizoctonia</i>   | Several crops   | Verdera Oy, Finland                         | USA, Germany, Canada, Finland           |
| <b><i>Rhizobium radiobacter</i></b> |   |   |   |   |   |
| Agrogal 130®                        | <i>R. radiobacter</i>   | Crown gall disease ( <i>Agrobacterium tumefaciens</i> )   | Ornamental, fruit and nut plants                                    | Probal Bioestimulantes Foliars Profer Chile | Chile                                   |
| Dygal®                              | <i>R. radiobacter</i>   | Crown gall ( <i>A. tumefaciens</i> )  | Ornamental and nut plants, pear, blueberry, grape, and other plants | AgBioResearch Ltd, Canada                   | Canada, New Zealand                     |
| Galltrol-A®                         | <i>R. radiobacter</i>   | Crown gall ( <i>A. tumefaciens</i> )  | Ornamental, fruit and nut plants                                    | AgBioChem, USA                              | USA                                     |
| Nogall™                             | <i>R. radiobacter</i>   | Crown gall ( <i>A. tumefaciens</i> )  | Ornamental, fruit and nut plants                                    | Becker Underwood Pty Ltd., Australia        | Australia, USA                          |

| Product                     | Bioagent / mode of action | Diseases / target pathogens  | Crop   | Company                        | Registered and commercialized              |
|-----------------------------|---------------------------|--|--|--------------------------------|--|
| <i>Pseudomonas spp.</i>     |                           |  |  |                                |  |
| Bio-save® 10/11             | <i>P. syringae</i>        | Blue mold ( <i>Penicillium expansum</i> ), green mold ( <i>Penicillium digitatum</i> ), gray mold ( <i>B. cinerea</i> ), mucor rot ( <i>Mucor piriformis</i> ), Fusarium dry rot ( <i>Fusarium sambucinum</i> ), helminthosporiosis ( <i>Helminthosporium spp.</i> )                   | Apple, pear, citrus, berries, sweet potato, potato | Jet Harvest Solutions, USA     | USA  |
| Cedomon®                    | <i>P. chlororaphis</i>    | Seedborne disease  | Barley and oats                                    | Lantmännen BioAgri AB, Sweden  | Italy, Finland, Sweden, Denmark, Poland    |
| Cerall®                     | <i>P. chlororaphis</i>    | <i>Tilletia caries</i> , <i>Septoria nodorum</i> and <i>Fusarium spp.</i>  | Wheat, rye and triticale                           | Lantmännen BioAgri AB, Sweden  | Sweden, Finland, Swiss, Austria, Lithuania |
| Spot-Less Biofungicide®     | <i>P. aureofaciens</i>    | Anthraxnose ( <i>Colletotrichum graminicola</i> ), root rot ( <i>Pythium aphanidermatum</i> ) and dollar spot ( <i>Sclerotinia homeocarpa</i> )  | Turf   | Turf Science Laboratories, USA | USA  |
| <i>Burkholderia cepacia</i> |                           |  |  |                                |  |
| Botrycid®                   | <i>B. cepacia</i>         | Soilborne pathogens ( <i>Rhizoctonia</i> , <i>Thielaviopsis</i> , <i>Verticillium</i> , <i>Fusarium</i> and <i>Pythium</i> ), disease caused by <i>Botrytis</i> , <i>Mycosphaerella</i> , <i>Erwinia</i> , <i>Xanthomonas</i> , <i>Agrobacterium</i> and <i>Ralstonia solanacearum</i> | Several crops                                      | Safer Agrobiologicos Colombia  | Colombia                                   |

Table 1. Bacterial biopesticides commercialized for the control of plant pathogens.

### 3.1 *Bacillus thuringiensis* as insecticide

The best-known *Bacillus* species used as a biopesticide is *B. thuringiensis* (Bt). This bacterium produces the proteins Cry and Cyt which are highly toxic to insects but not to mammals or for the environment. This contributes to explain the early use of this biopesticide which was first applied in 1938 (Sanahuja et al., 2011). Cry toxins are part of the structure of the *B. thuringiensis*' spores. When these bacterial spores are ingested by an insect, the Cry proteins act through pore formation in the gut wall of the animal allowing the bacteria that emerge from the spores to feed on the contents of the insect's body cavity. This generates a new bacterial population and thus a new source of spores after the death of the insect (Sanahuja et al., 2011).

Bt toxins are highly specific regarding their mode of action. The proteins are present in the spore in an inactive form but cleavage in the insect gut renders them toxic. Key factors for this event are the presence of specific proteases and an alkaline environment. This and the fact that toxicity also requires the presence of specific receptors in the insect's gut explains why the toxins are only effective on a small host range and thus often have limited effect on non target populations. Major insect families which can be controlled with Cry/Cyt toxins are Coleoptera, Lepidoptera and Diptera. Much of the research done on this biopesticide focuses on discovering new Cry toxins or combination of toxins to cover new ranges of insect pests. A diversity of products based on Bt toxins are being used on many crops and Bt toxins are even used to limit mosquito population in the context of malaria (Sanahuja et al., 2011). The mode of action of Cry also implies that the toxins must be present on the plant parts eaten by the target insect. Use in field is actually limited by the fact that the active

agent may be rapidly degraded or washed off the leaves surface. Pulverization has thus to be repeated more or less frequently depending on environmental conditions. Many efforts have been made with variable success to limit this drawback but the ultimate solution to this problem came in the form of GMO crops capable to produce the Bt toxins by themselves. These plants represent approximately 36% of all biotech crops cultured in 2009 corresponding to more than 50 million hectares. The advantage of the GMO crops compared to pulverization is the reduced loss of active component into the environment limiting need for repeated pulverization and potentially reducing pollution as, after plant death, crop residue does not seem to impact soil health indicators like earthworm populations. By contrast, risks are the emergence of new pests and the development of resistant pest populations. The risk of new pest emergence is mainly driven by the specificity of the toxin. The target pest being suppressed this opens an ecological space for a new pest. Efforts are made to limit resistant population development mainly by using combinations of Cry toxins and/or by planting non-resistant plants near GMO parcels thereby limiting selective pressure. Meanwhile, GMO crops continue to be a subject of intense political debate (Sanahuja et al., 2011).

### 3.2 Other *Bacillus* spp. for the control of multiple diseases

*B. thuringiensis* is an important microbial pesticide that has been the topic of recent reviews. In this chapter, we will better illustrate the agronomic interest of other *Bacillus* species. A variety of strains of *Bacillus* and particularly *B. subtilis* are currently commercialized as biopesticides (Table 1) and numerous studies are in support of the great potential of such strains at controlling multiple diseases occurring on a wide range of host plant species (Table 2). This potential is further illustrated in the following concrete examples.

| Crops        | Pathogens  | References  |
|--------------|--|---|
| <b>Fungi</b> |  |   |
| Abricot      | <i>Moniliana laxa</i>  | (Altindag et al., 2006)   |
| Alfalfa      | <i>Fusarium graminearum</i>  | (Chan et al., 2003)   |
| Amaranthus   | <i>Choanephora cucurbitarum</i>  | (Emoghene & Okigbo, 2001)   |
| Apple        | <i>Botrytis cinerea</i>  | (Touré et al., 2004)  |
| Avocado      | <i>Rosellinia necatrix</i>   | (Cazorla et al., 2007)  |
|              | <i>Colletotrichum gloeosporioides</i><br><i>Lasioidiplodia theobromae</i> , <i>Dothiorella aromatica</i><br><i>Thyronectria pseudotrichia</i><br><i>Phomopsis perseae</i><br><i>Pseudocercospora purpura</i> | (Demoz & Korsten, 2006)<br><br><br><br><br><br><br><br><br><br>(Korsten et al., 1997) |
| Banana       | <i>Pseudocercospora musae</i><br><i>Colletotrichum musae</i>   | (Fu et al., 2010)   |

| Crops       | Pathogens   | References   |
|-------------|---|--|
| Bean        | <i>Uromyces phaseoli</i>  | (Baker et al., 1985)<br>(Bettiol & Varzea, 1992)   |
| Beet        | <i>Cercospora beticola</i><br><i>Pythium spp.</i>   | (Collins & Jacobsen, 2003)   |
| Blueberry   | <i>Monilinia vaccinii-corymbosi</i>   | (Dedej et al., 2004)<br>(Scherm et al., 2004)  |
| Carrot      | <i>Alternaria dauci</i>   | (Hernandez-Castillo et al., 2006)  |
| Cauliflower | <i>Pythium ultimum</i>  | (Abdelzaher, 2003)   |
| Citrus      | <i>Colletotrichum acutatum</i><br><i>Guignardia citricarpa</i><br><i>Phytophthora citrophthora</i><br><i>Phytophthora parasitica</i><br><i>Penicillium digitatum</i>              | (Kupper, 2009)<br>(Amorim & Melo, 2002)<br><br>(Leelasuphakul et al., 2008)                                |
| Chir-pine   | <i>Macrophomina phaseolina</i>  | (Singh et al., 2008)   |
| Coffee      | <i>Hemileia vastatrix</i>   | (Bettiol & Varzea, 1992)<br>(Haddad et al., 2009)  |
| Corn        | <i>Fusarium moniliforme</i><br><i>Fusarium verticillioides</i><br><i>Aspergillus flavus</i><br><i>Fusarium solani</i><br><i>Pythium spp.</i><br><i>Rhizoctonia solani</i>         | (Bacon et al., 2001)<br>(Cavaglieri et al., 2005)<br><br>(Nesci et al., 2005)<br>(Cavaglieri et al., 2005) |
| Cotton      | <i>F. oxysporum</i>   | (Gajbhiye et al., 2010)  |
| Cucumber    | <i>Pythium aphanidermatum</i><br><i>Phytophthora nicotianae</i><br><i>R. solani</i><br><i>Phomopsis spp.</i><br><i>Colletotrichum lagenarium</i><br><i>Sphaerotheca fuliginea</i> | (Grosch et al., 1999)<br><br>(Kita et al., 2005)<br>(Ongena et al., 2005)<br>(Bettiol et al., 1997)        |
| Grape       | <i>Eutypa lata</i><br><i>B. cinerea</i><br><i>F. oxysporum</i><br><i>Botryodiplodia theobromae</i>  | (Ferreira et al., 1991)<br><br>(Rodgers, 1989)<br><br>(Swain et al., 2008)                                 |
| Lentil      | <i>Fusarium oxysporum f.sp. lentis</i>  | (El-Hassan & Gowen, 2006)  |
| Lettuce     | <i>P. aphanidermatum</i>  | (Corrêa et al., 2010)  |
| Litchi      | <i>Peronophythora litchi</i>  | (Jiang et al., 2001)   |

| Crops        | Pathogens   | References   |
|--------------|---|--|
|              | <i>Alternaria alternata</i><br><i>Cladosporium spp.</i>   | (Sivakumar et al., 2007)   |
| Mango        | <i>Oidium mangiferae</i>  | (Nofal & Haggag, 2006)   |
| Mellon       | <i>Podosphaera fusca</i>  | (Romero et al., 2007b)   |
| Mustard      | <i>Alternaria brassicae</i>   | (Sharma & Sharma, 2008)  |
| Nectarine    | <i>Monilinia laxa</i>   | (Casals et al., 2010)  |
| Oilseed rape | <i>Sclerotinia sclerotiorum</i>   | (Hu et al., 2005)<br>(Yang et al., 2009)   |
| Potato       | <i>R. solani</i>  | (Brewer & Larkin, 2005)<br>(Schmiedeknecht et al., 1998)   |
| Pear         | <i>Monilinia fructicola</i>   | (Pusey & Wilson, 1984)   |
| Peach        | <i>M. laxa</i><br><i>M. fructicola</i>  | (Casals et al., 2010)<br>(McKeen et al., 1986)<br>(Fan et al., 2000)                                       |
| Pepper       | <i>Phytophthora capsici</i><br><i>R. solani</i><br><i>P. capsici</i><br><i>P. aphanidermatum</i>            | (Ahmed et al., 2003)<br><br>(Lee et al., 2008)<br>(Nakkeeran et al., 2006)                                 |
| Pinus        | <i>Ophiostoma picea</i><br><i>M. phaseolina</i>   | (Silo-Suh et al., 1998)<br>(Singh et al., 2008)  |
| Rice         | <i>Aspergillus flavus</i><br><i>Pyricularia oryzae</i><br><i>R. solani</i>                                  | (Reddy et al., 2009)<br>(Bettiol & Kimati, 1990)<br>(Yang et al., 2009)                                    |
| Rose         | <i>B. cinerea</i>   | (Tatagiba et al., 1998)  |
| Strawberry   | <i>B. cinerea</i><br><i>Podosphaera aphanis</i>   | (Helbig & Bochow, 2001)<br>(Pertot et al., 2008)   |
| Soybean      | <i>Septoria glycines</i><br><i>F. oxysporum</i><br><i>F. graminearum</i><br><i>Sclerotinia sclerotiorum</i> | (Mantecon, 2008)<br>(Zhang et al., 2009)   |
| Sorghum      | <i>P. ultimum</i>   | (Idris et al., 2008)   |
| Tobacco      | <i>P. aphanidermatum</i><br><i>Cercospora nicotiana</i>   | (Maketon et al., 2008)   |
| Tomato       | <i>F. oxysporum</i><br><i>Fusarium semitectum</i><br><i>F. oxysporum f.sp. lycopersici</i>                  | (Chebotar et al., 2009)<br>(Nihorimbere et al., 2010)<br>(Abd-Allah et al., 2007)<br>(Baysal et al., 2008) |

| Crops            | Pathogens   | References   |
|------------------|---|--|
|                  | <i>P. aphanidermatum</i>  | (Jayaraj et al., 2005)<br>(Ongena et al., 2005)                              |
|                  | <i>R. solani</i>  | (Kondoh et al., 2000)<br>(Kondoh et al., 2001)<br>(Montealegre et al., 2003) |
| Wheat            | <i>Gaeumannomyces graminis var. tritici</i>   | (Liu et al., 2009)   |
| Yam              | <i>F. oxysporum</i><br><i>Botryodiplodia theobromae</i><br><i>B. theobromae</i><br><i>Fusarium moniliforme</i><br><i>Penicillium sclerotigenum</i><br><i>Rhizoctonia spp.</i> | (Swain et al., 2008)<br><br>(Okigbo, 2003)                                   |
| <b>Bacteria</b>  |   |  |
| Arabidopsis      | <i>Pseudomonas syringae</i>   | (Bais et al., 2004)  |
| Brassica         | <i>Xanthomonas campestris pv. campestris</i>  | (Wulff et al., 2002)   |
| Mulberry         | <i>Ralstonia solanacearum</i>   | (Ji et al., 2008)  |
| Soybean          | <i>X. campestris pv. glycines</i>   | (Salerno & Sagardoy, 2003)   |
| Tobacco          | <i>R. solanacearum</i>  | (Maketon et al., 2008)   |
| Tomato           | <i>Xanthomonas euvesicatoria</i><br><i>Xanthomonas perforans</i>  | (Roberts et al., 2008)   |
| <b>Nematodes</b> |   |  |
| Tomato           | <i>Meloidogyne</i><br><i>Meloidogyne incognita</i>  | (Araújo & Marchesi, 2009)<br>(Siddiqui & Futai, 2009)                        |
| Soybean          | <i>Heterodera glycines</i>  | (Araújo et al., 2002)  |

Table 2. Potential of *Bacillus subtilis* for the control of plant pathogens.

Bean rust caused by *Uromyces appendiculatus* can cause severe damages when it occurs early in culture. Relying on genetic resistance is not really successful due to the large variability of the causal agent and thus the control of the disease was traditionally achieved by using chemical fungicides. In greenhouse assays, Baker and collaborators tested the soil originating strain APPL-1 of *Bacillus subtilis*. Treatment with this isolate decreased the number of pustules of bean rust by 95%, when applied on plants 2 to 120 h before the inoculation of uredospores of *Uromyces appendiculatus* (Baker et al., 1983). In field conditions, reduction of at least 75%, in the occurrence of rust, was observed upon three weekly applications of the strains APPL-1 and PPL-3 (Baker et al., 1985). Centurion (1991) obtained a reduction of 80 to 100% in the number of pustules of rust by applying *Bacillus subtilis* strain

W401 in greenhouse assays (Centurion, 1991). Mizubuti (1992) observed significant reduction in the germination of uredospores of *Uromyces appendiculatus* by five strains of *Bacillus subtilis*, and a reduction in the number of pustules per leaf when the strains were applied 48 h before inoculation with the pathogen in the greenhouse (Mizubuti, 1992). Bettiol and collaborators observed that extracts of *B. subtilis*, obtained by the precipitation of metabolites with ammonium sulphate or by acidification at pH 2.0, at a concentration of 1000 ppm totally inhibited the germination of urediniospores of *U. appendiculatus*, and controlled the rust (84%) when sprayed on bean leaves (Bettiol et al., 1992). Nowadays, the product Bio safe® (Table 1) with cells of *B. subtilis* is used to control anthracnose in bean and Asian soybean rust. In recent years, the product has been used in integrated pest management for these crops.

Coffee leaf rust (*Hemileia vastatrix*) is the most important coffee disease in Brazil. Bettiol and Varzea observed that cell suspensions of *Bacillus subtilis* strains AP-3 and AP-150 totally inhibited urediniospore germination of various races of *Hemileia vastatrix* (Bettiol et al., 1992). Spraying of sterilized and unsterilized *Bacillus* cell suspensions on detached leaves of coffee cv Caturra allowed reduction of the number of lesions by 72% to 87% depending on the pathogen race. The same disease control trend was observed when these strains were sprayed on whole coffee plants. Under commercial conditions, Haddad and collaborators (2009) also showed that the strain B157 of *Bacillus* sp. can be considered a potential biocontrol agent for coffee leaf rust in organic crop systems in Brazil (Haddad et al., 2009).

As third example, a product formulated with *Bacillus subtilis* and *Bacillus licheniformis*, has been used for the control of diseases caused by the nematode pathogens *Meloidogyne incognita*, *Meloidogyne javanica*, *Pratylenchus brachyurus* and *Pratylenchus coffeae* on potatoes and carrots in Brazil. In 2008, more than 12,000 kg of this product were commercialized only for the treatment of potato and carrot. 5 - 10 kg/ha of the product, with  $2 \times 10^{10}$  CFU/g, were applied by irrigation and other ways. The cost was approximately US\$ 160-300/ha. This product has replaced nematicides.

#### **4. Deciphering the mechanisms involved in biocontrol of plant diseases by *Bacillus***

By taking benefits from the nutrients constantly released from roots or leaves of growing plants, beneficial bacterial strains efficiently colonize leaf surfaces and root systems and their surrounding soil layer. In turn, they beneficially influence the plant by protecting it from infection by plant pathogens via three main mechanisms: competition for ecological niche/substrate, production of inhibitory allelochemicals, and induction of systemic resistance in host plants. It should be noted that none of these mechanisms described below are necessarily mutually exclusive, and frequently several modes of action are exhibited by a single biocontrol agent. In the next sections, we mainly consider beneficial microbes introduced in soil but the same principles and mechanisms apply for isolates used to combat foliar diseases.

##### **4.1 Competition for niche and nutrients**

Competition for resources such as nutrients and oxygen occurs generally in soil among soil-inhabiting organisms. For biocontrol purpose, it occurs when the antagonist directly competes against pathogens for these resources. Root inhabiting microorganisms compete

for suitable sites at the root surfaces. Competition for nutrients, especially for carbon, is assumed to be responsible for the well-known phenomenon of fungistasis, characterizing the inhibition of fungal spore germination in soil (Alabouvette et al., 2006). Given the relatively low abundance of substrates in the rhizosphere, the efficiency of nutrient uptake and catabolism by bacteria is a key factor in competitiveness. Competition for trace elements, such as iron, copper, zinc, manganese etc., also occurs in soils. For example, iron is an essential growth element for all living organisms and the scarcity of its bio-available form in soil habitats results in a furious competition (Loper & Henkels, 1997). Siderophores, low molecular weight compounds with high iron affinity, are produced by some microorganisms (and also by most biocontrol agents) to solubilize and competitively acquire ferric ion under iron-limiting conditions, thereby making iron unavailable to other soil microorganisms which cannot grow for lack of it (Haas & Défago, 2005; Loper & Henkels, 1997). Suppression of soilborne plant pathogens through competition for niche and nutrients has been demonstrated in some instances for some beneficial bacteria such as *Pseudomonas* (Haas & Défago, 2005). Experimental proof concerning *Bacillus* is scarce but these competitive phenomena should also occur with this bacterium given its natural rhizosphere competence.

## 4.2 Direct inhibition of phytopathogens

### 4.2.1 Antibiosis

Members of multiple *Bacillus* species such as *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, and *B. pumilus* are known as very efficient producers of antibiotic molecules. *Bacillus subtilis* has an average of 4-5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds (Stein, 2005). In strain FZB42, which is proposed as a paradigm for plant-associated *Bacillus amyloliquefaciens* as well as in other isolates, an even larger part of the genome (~8%) is seemingly involved in antibiotic synthesis (Arguelles-Arias et al., 2009; Chen et al., 2009; Ruckert et al., 2011). Among the vast array of biologically active molecules synthesized by *Bacillus*, some have been reported for their inhibitory activity against plant pathogens and this antagonistic activity or antibiosis is probably the best-known and the most important mechanism used to limit pathogen invasion in host plant tissues.

*B. cereus* UW85 produces two fungistatic antibiotics, zwittermicin A and kanosamine, which are suggested to contribute to the suppression of damping-off disease of alfalfa caused by *Phytophthora medicaginis* (Silo-Suh et al., 1994). Zwittermicin A may also control the fruit rot of cucumber (Smith et al., 1993) and suppress other plant diseases (Silo-Suh et al., 1998). Bacillaene, difficidin and macrolactin are polyketides displaying a broad spectrum of antibacterial activities that may be involved in the biocontrol activity of the producing strain such as in the case of fire blight, a serious disease of orchard trees caused by *Erwinia amylovora* (Chen et al., 2009). The phosphono-oligopeptide rhizoctin produced by *B. subtilis* also displays antifungal and nematocidal activities, but does not retain any bactericidal properties (Borisova et al., 2010). Peptide compounds represent the predominant class of *Bacillus* antibiotics. They are of various sizes, may be composed entirely of amino acids but some contain other residues. Cyclic or linear oligopeptides, basic peptides and aminoglycoside antibiotics usually occur (Stein, 2005). Low molecular weight and hydrophobic or cyclic structures, with unusual constituents like D-amino acids, are also

common characteristics of peptide antibiotics normally synthesized by *Bacillus*. Moreover, they are generally resistant to hydrolysis by peptidases and proteases of animal and plant origin. *Bacillus brevis* (*BreviBacillus brevis*) and *Bacillus polymyxa* (*PaeniBacillus polymyxa*) produce gramicidin S and polymyxin B peptide antibiotics that strongly inhibited *Botrytis cinerea* germination *in vitro* but also exhibited high activity under natural field conditions against the *Botrytis* grey mould disease caused by this fungus on strawberry (Haggag, 2008). Another group of peptide antibiotics usually produced by *Bacillus subtilis* are lantibiotics (Stein, 2005). These compounds display strong antibacterial properties against gram-positive bacteria but their involvement in the biocontrol activity of plant-associated *Bacillus* isolates has not been clearly demonstrated so far. More simple molecules, such as the dipeptide bacilysin (L-Ala linked to the non-proteinogenic amino acid L-anticapsin), also retain strong bactericidal effect and are seemingly involved in the control of some plant pathogens (Chen et al., 2009).

A major class of *Bacillus* peptide antibiotics are cyclic lipopeptides (cLPs) which may vary in the type and sequence of amino acid residues, the nature of the peptide cyclization and in the nature, length and branching of the fatty acid chain (Ongena & Jacques, 2008). In various species of *Bacillus*, the three main families are surfactins, iturins and fengycins. They encompass structural variants depending on the genetic background of the considered strain. Surfactins are heptapeptides interlinked with a  $\beta$ -hydroxy fatty acid to form a cyclic lactone ring structure. Iturins, with 7 variants including bacillomycins and mycosubtilin, are also heptapeptides but are linked to a  $\beta$ -amino fatty acid chain with a length from C<sub>14</sub> to C<sub>17</sub>. Fengycins A and B, also called plipastatins, are lipodecapeptides with an internal lactone ring in the peptidic moiety and with a  $\beta$ -hydroxy fatty acid chain (C<sub>14</sub> to C<sub>18</sub>) that can be saturated or not. Beside these three main families, other classes of bioactive lipopeptides synthesized by *Bacillus* species have been identified (Hathout et al., 2000; Lee et al., 2007).

Each family of *Bacillus* cLPs displays specific antibiotic activities and may thus be differentially involved in the antagonism of the various plant pathogens. In the case of soilborne diseases, iturin A produced by *B. subtilis* RB14 was involved in the control of damping-off of tomato (a seedling disease) caused by *Rhizoctonia solani* (Asaka & Shoda, 1996). Overexpression of mycosubtilin in *B. subtilis* ATCC 6633 also led to a significant reduction of seedling infection by *Pythium aphanidermatum* (Leclère et al., 2005). As examples in the control of phyllosphere diseases, a contribution of both iturins and fengycins was shown in the antagonism of *B. subtilis* toward *Podosphaera fusca* infecting melon leaves (Romero et al., 2007a). This was notably demonstrated by showing the strong inhibitory effect of these cLPs on *P. fusca* conidia germination, and by recovering cLPs from bacterial-treated leaves and using cLP-deficient mutants. In the protection against post harvest diseases, *Bacillus subtilis* strain GA1, which efficiently produces cLPs from the three families and notably a wide variety of fengycins, protected wounded apple fruits against gray mold disease caused by *Botrytis cinerea*. The role of fengycins was demonstrated by the very effective disease control provided by treatment of fruits with cLPs-enriched extracts and by *in situ* detection of fengycins in inhibitory amounts (Touré et al., 2004). To further illustrate the broad range of fungal targets, fengycins were also reported for their antagonistic activity against *Fusarium graminearum* (Wang et al., 2007), and iturins for their inhibitory effect towards the anthracnose-causing agent *Colletotrichum dematium* (Hiradate et al., 2002), *Penicillium roqueforti* (Chitarra et al., 2003), *Aspergillus flavus* (Moyne et al., 2001), *Rhizoctonia solani* (Yu et al., 2002), wood-staining fungi (Velmurugan et al., 2009) and nematophagous fungi (Li et al., 2007). In some instances, the fungitoxic activity was clearly related to the

permeabilization of spore/conidia therefore inhibiting germination or alternatively to hyphal cell perturbation. As revealed by transmission electron microscopy techniques, both phenomena most probably result from membrane damaging by the cLPs (Chitarra et al., 2003; Etchegaray et al., 2008; Romero et al., 2007a).

A few studies have revealed some insecticide activity of cLPs from *B. subtilis*. Surfactin and iturin were described for their antagonistic effect against fruit fly *Drosophila melanogaster* (Assie et al., 2002) and cLPs contained in a crude extract were efficient at inhibiting the development of larvae of the mosquito *Culex quinquefasciatus* (Das & Mukherjee, 2006). Although active doses are quite high (approx. 200  $\mu$ M) and mechanisms underpinning such biocidal effect have not yet been investigated, treatments with cLPs are presented as possible alternatives for the use of the endotoxin producer *B. thuringensis* in the biocontrol of insects for which this bacterium is not efficient.

#### 4.2.2 Other inhibitory mechanisms

Distinct from antibiosis that involves low-molecular weight compounds and does not require physical contact, predation/parasitism is also an important mechanism used by some biocontrol microorganisms, mainly fungi such as *Trichoderma*. It is based on enzymatic destruction of the fungal pathogen cell wall. The ability of bacteria to parasitize and degrade spores or hyphae of pathogens through the production of various cell-wall degrading enzymes has also been suggested (Whipps, 2001). As examples, isolates related to *Bacillus ehimensis* (Hoster et al., 2005) produce chitin-degrading enzymes while *Bacillus subtilis* AF1 displays some fungitoxicity through the secretion of *N*-acetyl glucosaminidase and glucanase (Manjula & Podile, 2005). Some more specific pathogen-biocontrol strain interactions leading to pathogen restriction were reported such as interference with biofilm formation, inactivation of pathogen germination factors and degradation of pathogenicity factors such as toxins but will not be detailed here.

#### 4.3 Plant resistance triggering

The isolation of some PGPR strains efficient in biocontrol but lacking the ability to exert any antagonistic activity toward pathogens shed new light on the diversity of their modes of action and suggested that such strains may activate defense systems in the host plant. This stimulation of the plant immune system represents one of the most newly discovered aspects of plant-microbe interactions (Bakker et al., 2007). Some isolates are indeed able to reduce disease through the stimulation of a primed state in the host plant which allows an accelerated activation of defense responses upon pathogen attack, leading to an enhanced resistance to the attacker encountered (Conrath et al., 2006). Conclusive evidence for the role of induced systemic resistance (ISR) in disease reduction by a given bacterium tested on a particular pathosystem is obtained by verifying the spatial separation of the pathogen and the resistance-inducing agent in order to exclude any direct antagonistic interaction. ISR can be globally viewed as a three-step process involving sequentially i) the perception by plant cells of elicitors produced by the inducing agents that initiates the phenomenon, ii) signal transduction that is needed to propagate the induced state systemically through the plant and iii) expression of defense mechanisms *stricto sensu* that limit or inhibit pathogen penetration into the host tissues (Van Loon, 2007). Defense molecules include phytoalexins, pathogenesis-related (PR) proteins (such as chitinases,  $\beta$ -1,3-glucanases, proteinase inhibitors, etc.) and lignin for reinforcement of cell walls (Van Loon, 2007). Cell wall thickenings, wall appositions or rapid death of the injured plant cells resulting in necrosis of

the immediate adjacent tissues are barriers which cut the pathogen off its nutrients and contribute to slowing down the fungal invasion (Lugtenberg et al., 2002).

The list of bacteria identified as ISR inducers has grown rapidly over the last two decades and includes Gram-negative bacteria such as members of the *Pseudomonas* and *Serratia* genera but also Gram-positive bacteria and more particularly *Bacillus* spp. (Bent, 2006; Kloepper et al., 2004). Rhizobacteria-mediated ISR can occur in many dicotyledonous and monocotyledonous plant species. By analogy with the pathogen-induced SAR, protection afforded through ISR is quite non-specific regarding the nature of the infectious agent. Because of its systemicity, the enhanced defensive capacity is expressed in roots as well as in leaves. Control of diseases caused by fungi, bacteria, and viruses has been demonstrated thoroughly but ISR may also be a successful strategy in management of nematode and insect pests in several crops. Rhizobacteria-mediated ISR does not confer a total protection against pathogen infection but as the phenomenon is long-lasting and not conducive for development of pathogen resistance (multiplicity and variety of induced defense pathways), ISR-based biocontrol strategies are promising and some trials were successfully performed under field conditions.

Volatile compounds such as 2,3-butanediol (Ryu et al., 2004) and lipopeptides are the sole compounds formed by *Bacillus* spp. that were identified as elicitors of ISR. The potential of *Bacillus* cLPs as plant resistance inducers was demonstrated by testing pure surfactins and fengycins that provided a significant induced protective effect similar to the one induced by living cells of the producing strain (*B. amyloliquefaciens* S499). In a complementary approach, experiments conducted on bean and tomato showed that overexpression of both surfactin and fengycin biosynthetic genes in the naturally poor producer *B. subtilis* strain 168 was associated with a significant increase in the potential of the derivatives to induce resistance. Moreover, the macroscopic disease reduction induced by the surfactin overproducer was associated with defense-related metabolic changes in the host plant tissues (Ongena et al., 2007).

## 5. Improving biopesticides and conclusive statements

Microbial biopesticides and *Bacillus* based products in particular improve plant health through many mechanisms and gain increasing interest for commercial application as exemplified above. Unfortunately, these products often offer only partial protection against pathogen and pest attacks. Another weakness of microbial biopesticides is their inconsistent effect. As the active ingredient is a living organism, its efficacy is more strongly dependant on application conditions compared to conventional pesticides. The activity of the beneficial organism depends on its global ecology, in other words the interactions between the beneficial organism, the plant host, the pathogen and the biotic and abiotic environmental parameters (Butt et al., 1999; Fuentes-Ramirez & Caballero-Mellado, 2006).

Unfortunately, our knowledge about the ecology of most beneficial microorganisms used today is still poor, limiting rational field applications. In a more "critical" point of view, we will point out that, at least in the case of *Bacillus*, one of the main causes of this lack of information is the often-observed deficiency in connections between field trials and more controlled laboratory experiments. For instance, it is often speculated that the frequent occurrence of *B. subtilis* in its natural environment might be due to the selective advantage conferred by the panoply of bioactive metabolites that it may produce. However, even if some *B. subtilis* strains are well equipped genetically to produce a vast array of antibiotics,

only a limited part of this antibiotic-devoted genetic background may be readily expressed in soil and thus, only a part of this arsenal may be actually produced under natural conditions. The recent developments in biotechnology and analytical chemistry open the doors to new approaches for investigation and new tools to study *in situ* antibiotic production by valuable strains in their ecological niche. This will surely contribute to enhance our knowledge about *Bacillus* fitness in natural living conditions which is a crucial point for optimizing biocontrol strategies using this organism.

There are many other possible approaches for the improvement of biopesticide efficacy and consistency of protection. Strain selection also probably deserves further improvement. The methods through which this selection is conducted are subject of debate. *In vitro* screening, though easy to implement, often includes the drawback of not taking into account environment conditions for field application. In order to optimize selection process, a middle path has to be found between more realistic and easier selection methods. A potential long-term solution could be to invest more in fundamental research linking field observations to readily testable parameters.

In the context of biopesticide improvement, one must consider when possible the whole agricultural system. A key to improve plant health and growth is to find adequate combinations between biopesticides, chemical pesticides, plant fertilization, agricultural practices like different types of tillage etc. as embodied by integrated pest management (Chakraborty et al., 2010; Dukare et al., 2011; Kumar et al., 2010). Moreover, when implementing biopesticides in integrated pest management, the biological product must be compatible with conventional pesticides. This parameter is of particular importance when the applications of the biological and chemical ingredients occur simultaneously for example in seed treatment or in combined foliar sprays.

The simultaneous implementation of several active ingredients in one commercial product is certainly a way to guarantee the global efficacy under varying conditions. A microbial strain can be used together with other strains, with natural extracts or other none chemically transformed products or with chemical pesticides (Shanmugam & Kanoujia, 2011; Liu et al., 2011; Akila et al., 2011; Kondoh et al., 2001). These combinations are generally more effective and reliable. For example, combinations of strains can be selected to broaden pathogen spectrum by blending strains with distinct action mechanisms, or to enhance reliability by mixing isolates with different ecological competences (Jijakli, 2003; Ramamoorthy et al., 2001). Moreover, the combination of strains can induce synergic effects improving biocontrol. An advantage of combining products is also that several treatments are applied at once reducing labor for the farmer. The main setbacks of combination products are: potential antagonisms between the active ingredients (even between two strains) (Whipps, 2001) and more fastidious homologation procedures as the ingredients contained in the mix must pass legal tests individually. This is mainly a problem in Europe where pesticide legislation is very strict. In the U.S., biopesticide homologation is simpler as the one used for classic pesticides as these first products are considered less dangerous (Jijakli, 2003).

Formulation and application methods are also key issues influencing the efficacy of commercial products and research on these topics should be focused on specific environmental applications. For example, in the case of formulation, a possibility is the addition of molecules that favor the adhesion of the bacteria to fruit or leaves when used as a spray (Rabindran & Vidhyasekaran, 1996). Another option could be to combine the strain with a substrate like chitin which may stimulate biocontrol activity (Ahmed et al., 2003).

In conclusion, we can say that microbial biopesticides have great potential that is and should be even more used to make the future agriculture more sustainable. Much work has been done but a lot is still to do for scientists and industrials, to improve reliability and efficacy of these products and keep gaining an increasing market share.

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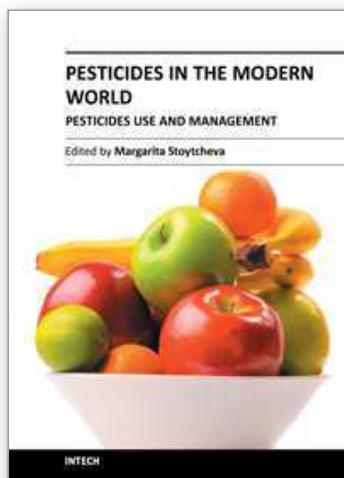
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## **Pesticides in the Modern World - Pesticides Use and Management**

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This book brings together issues on pesticides and biopesticides use with the related subjects of pesticides management and sustainable development. It contains 24 chapters organized in three sections. The first book section supplies an overview on the current use of pesticides, on the regulatory status, on the levels of contamination, on the pesticides management options, and on some techniques of pesticides application, reporting data collected from all over the world. Second section is devoted to the advances in the evolving field of biopesticides, providing actual information on the regulation of the plant protection products from natural origin in the European Union. It reports data associated with the application of neem pesticides, wood pyrolysis liquids and bacillus-based products. The third book section covers various aspects of pesticides management practices in concert with pesticides degradation and contaminated sites remediation technologies, supporting the environmental sustainability.

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