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## **Durability of efficacy of microbial bioprotectants against plant diseases**

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### 1 Introduction

Much research effort has been dedicated in the last 50 years to the development of microbial bioprotectants against plant pathogens, resulting in the registration of an increasing number of commercial products. A recent study by van Lenteren et al. (van Lenteren et al., 2018) has identified 91 microbial bioprotectants (49 filamentous fungi, yeasts or oomycetes, 37 bacteria and 5 viruses including bacteriophages) registered against plant pathogens in Australia, Brazil, Canada, the European Union, Japan, New-Zealand and the United States of America. This number of microbial bioprotectants is probably underestimated due to the lack of data for some countries where biocontrol is experiencing increasing use, such as China, Russia or India. Moreover, a similar strain of a given microbe can be commercialized with different names, either because it is formulated differently or because it is distributed by different companies. The development of a microbial bioprotectant product involves the screening of a large number of microbial strains on the basis of different criteria (Köhl et al., 2011). A number of issues must be considered before a product is registered and placed on the market (Glare et al., 2012). They include technical issues, such as the study of environmental factors on the efficacy against the targeted plant pathogen, the development of methods for mass production of the

microorganism, the elaboration of a formulation of the product and the validation of its effect under commercial production conditions of the crop. They also include regulatory issues, related to toxicology and ecotoxicology and economic issues (Ravensberg, 2011).

A recurring problem encountered with microbial bioprotectants is their occasional inconsistent efficacy in the field (Nicot et al., 2011). This situation is generally attributed to microclimatic variations encountered in agricultural production conditions (e.g. temperature, relative humidity and radiation), a lack of ecological competence of the microbial bioprotectant (e.g. survival and colonization capacity), intrinsic characteristics of the product (e.g. variable production of metabolites or enzymes involved in the efficacy of the control) and/or the unstable quality of the bioprotectant product (e.g. shelf life).

However, possible loss of efficacy of a biocontrol agent may arise if isolates with low susceptibility occur in natural populations of plant pathogens and become abundant as a result of the selection pressure exerted by the products applied by farmers. Though the resistance of plant pathogens to microbial bioprotectants is still understudied, recent work shows that the susceptibility of plant pathogens to microbial bioprotectants can be highly variable (Bardin et al., 2015). Moreover, cases of resistance (or reduced sensitivity) to microbial bioprotectants have been observed under commercial growing conditions among invertebrate pests (Siegwart et al., 2015). Thus, the diversity in the sensitivity of various strains of a pathogen to a microbial bioprotectant, and its ability to evolve towards resistance, must be considered to ensure effective and durable protection. This review highlights current knowledge concerning the erosion of microbial protection against plant pathogens and its possible consequences for field efficacy.

## 2 Durability of plant protection against pests

The durability of a control method for plant protection is defined as the persistence of its efficacy in space and time. It depends on the selection pressure exerted by the control method itself on populations of pests, as well as on the ability of these pests to adapt to this method.

Erosion of efficacy has been widely studied in the past for several plant protection methods. The durability of chemical control has, for instance, long been challenged by the frequent and recurrent emergence of resistance to fungicides in populations of various major plant pathogenic fungi (Brent and Hollomon, 2007; REX-Consortium, 2013). Likewise, the breakdown of plant varietal resistances, in

particular those linked to major resistance genes, is also well documented for plant pathogens and insect pests (McDonald and Linde, 2002; REX-Consortium, 2016). Cases of resistance (or reduced sensitivity) of plant pathogens to physical control methods have also been described. Thus, the phytopathogenic fungus *Botrytis cinerea* is able to become gradually accustomed to the inhibitory effect of UV-filtering films (Nicot et al., 2001). Filtering out the small amounts of near-UV radiation provided by natural light causes a sharp reduction in spore production by this fungus. After several successive generations under selection pressure (absence of near-UV radiation), sporulation of the fungus was shown to increase again. Although many pests are known for their ability to develop resistance to conventional methods of plant protection, only a few studies have explored their ability to overcome the effect of microbial bioprotectants.

Resistance (or lower susceptibility) in the field to microbial biocontrol agents or to metabolites produced by microbials has already been observed in arthropod pests (Siegwart et al., 2015). Resistance to one or several toxins of the most widely used microbial bioprotectant in the world, *Bacillus thuringiensis*, has, for example, been described several years after its market approval in three species of pests (*Plutella xylostella*, *Trichoplusia ni*, *Plodia interpunctella*). Under laboratory conditions, at least 27 insect species are capable of developing resistance to *B. thuringiensis* (Tabashnik, 1994). But resistance of pests to other microbial bioprotectants has also been pointed out. A striking example concerns the resistance of codling moth (*Cydia pomonella*) to the bioprotectant *C. pomonella* granulovirus. This resistance was detected jointly in France and in Germany in pome fruit orchards, after more than 10 years of intensive use of commercial products derived from an identical entomopathogenic viral isolate (Asser-Kaiser et al., 2007; Sauphanor et al., 2006). A very high resistance factor of 13 000 was observed (i.e. it took 13 000 times more virus particles to eradicate a population of resistant insects than to eradicate a population of susceptible insects). Under laboratory conditions, repeated treatments with this virus on the resistant population of *C. pomonella* made it possible to select an even higher resistance level, with a resistance factor of 60 000 (Berling et al., 2009).

To our knowledge, most of the proven cases of resistance of pests to microbial bioprotectants concern harmful insects. Although biological control against plant pathogens (bacteria, fungi, oomycetes, nematodes, viruses) has been widely studied in the past years leading to the marketing of numerous microbial bioprotectants, the durability of their efficacy is still poorly studied, and no case of resistance in the field has yet been described (Bardin et al., 2015). This lack of documented cases of resistance of plant pathogen to microbial bioprotectants may be explained by their still limited use in agriculture compared to conventional protection tools.

### 3 Main modes of action of microbial bioprotectants against plant pathogens and potential mechanisms of resistance developed by plant pathogens

The mechanisms underlying the protective effect of a given microbial bioprotectant are often only partially understood, although the number of scientific studies done on this subject is quite important. However, data available to date suggest that the protection of plants against harmful microorganisms by microbial bioprotectants is based on mechanisms linked to direct (antibiosis, hyperparasitism) and/or indirect (competition, induced resistance, interference with the mechanisms of pathogenesis) interactions with the pathogens (Alabouvette et al., 2009; Bardin and Pugliese, 2020; Haidar et al., 2016; Jacobsen, 2006; Köhl et al., 2019).

In the case of antibiosis, the antagonistic microorganism produces secondary metabolites that are toxic to the targeted plant pathogen. Metabolites produced at low concentrations may, for instance, inhibit spore germination, mycelial growth or the sporulation of fungal plant pathogens. There are many examples of bacteria and fungi producing compounds with an antimicrobial effect, including *Bacillus* sp., *Pseudomonas* sp., *Streptomyces* sp., *Trichoderma* sp. Toxic substances responsible for antibiosis have been characterized in different strains belonging to various microbial species, and in some cases, genes involved in the synthesis of some of these substances have been identified (Raaijmakers et al., 2002). Even if this mode of action is easily revealed in the laboratory by confrontation tests on a nutrient medium, knowledge of the conditions for the synthesis of antibiotic metabolites by the bioprotectant and of the quantities produced *in situ* is often not available. And finally, the ability of microbial bioprotectants to synthesize these molecules under the conditions of their use on crops is rarely demonstrated (Raaijmakers and Mazzola, 2012). The production of antibiotic compounds may depend on many characteristics of the environment in which the microorganism develops, such as the water potential, the pH of the medium, the temperature or the nutrient availability (Whipps, 1987). This mode of action is the closest to that of molecules used in chemical control. It can, therefore, be very effective in inhibiting the development of a plant pathogen but can raise concerns about the emergence of resistant strains. Plant pathogens are, for instance, known to have developed mechanisms of resistance to various toxic compounds produced by beneficial microorganisms (Duffy et al., 2003), particularly during their interaction in the soil (Raaijmakers et al., 2009). These mechanisms include excretion by active efflux and degradation of toxic compounds, interference with the regulation and biosynthesis of enzymes and metabolites produced by microbial bioprotectants.

In the case of hyperparasitism, the microbial bioprotectant parasitizes the plant pathogen. It specifically recognizes its target, enters these cells (e.g. bacterial cells, mycelium, spores or resting structures for fungal pathogens) and causes its destruction through the colonization of its organs. *Paraphaeosphaeria* (formerly *Coniothyrium*) *minitans* is, for example, a hyperparasitic fungus of the sclerotia of *Sclerotinia* sp., able to excrete enzymes degrading cell walls of the fungal pathogen (Whipps and Gerlagh, 1992). The fungus *Ampelomyces quisqualis* parasitizes specifically the plant pathogenic fungi that cause powdery mildew on several crops (Kiss et al., 2004). Bacteriophages can be considered as hyperparasites of bacteria as they proliferate in and eventually destroy the cells of their bacterial host (lytic phages). Phage-based commercial products are available worldwide against specific plant-pathogenic bacterial species (Zaczek et al., 2014; Buttimer et al., 2017). Direct contact of the microbial bioprotectant acting through hyperparasitism with the plant pathogen and the need for an adequate timing of the treatment are necessary to ensure satisfactory protection against the disease before too much damage is caused to the crop. Some plant pathogens have developed the ability to resist or repress the synthesis of cell wall-degrading enzymes required by microbial protectants to parasitize them (Bardin et al., 2015).

Competition for nutrients and space is another mode of action by which microbial bioprotectants can limit or even suppress the development of plant pathogens. For instance, competition is effective against phytopathogenic fungi whose spores need a source of nutrients to initiate their germination. In this case, the reduction in the nutrient concentration in the medium leads to a reduced rate of germinated spores of the pathogen and a slow-down of mycelial growth, thus decreasing the number of infections and the expansion of lesions (Blakeman and Fokkema, 1982). Some microorganisms (bacteria, yeasts, filamentous fungi) can inhibit the germination of conidia from fungal plant pathogens through competition for nutrients such as nitrogen, carbon or macro- or microelements present in the medium. For instance, the fungus *Trichoderma harzianum* inhibits the germination of *B. cinerea* conidia by competing for nutrients at the early stages of interaction (Zimand et al., 1996). Competition is not restricted to interactions during spore germination, it can also occur later in the pathogen growth process if the biocontrol agent is present in sufficient quantity. Competition for nutrients or for space has, for instance, been demonstrated for different yeast species against post-harvest fungal pathogens, including *B. cinerea* or *Penicillium. expansum* (Mercier and Wilson, 1994; Spadaro and Droby, 2016).

In addition to competition for carbohydrates or nitrogen, a well-studied case concerns competition for iron as well. Restricted iron availability can be a limiting factor for microbial growth. Various bacteria and fungi can synthesize high-affinity chelators named siderophores (Köhl et al., 2019). For example, isolates of *Pseudomonas* spp. compete for iron via siderophores, resulting in a reduction of populations

of pathogenic *Fusarium* in the rhizosphere (Lemanceau and Alabouvette, 1993; Lemanceau et al., 1992).

The resistance of pathogens to competitive mechanisms seems unlikely (Köhl et al., 2019). However, plant pathogens are continually subjected to stress related to their environment, such as nutrient limitation, and some strains may have developed the ability to rapidly exploit the nutritional resources available in their ecological niche. This type of ability could slow down the development of microbial bioprotectants by using more efficiently the resources and ultimately, influence the efficacy of biocontrol. For instance, in a study by Mazzola and Cook (1991), root infections by the plant pathogen *Pythium irregulare*, which causes root rot of wheat, significantly reduce the population of *Pseudomonas fluorescens* strains introduced in the soil for its control. The question of the ecological competence of biocontrol agents (ability to survive and develop in a given ecological niche) is, therefore, essential to ensure good efficacy against the targeted pathogen.

Many microorganisms have the ability to induce or to prime defense mechanisms in plants after a signal recognition phase (Pieterse et al., 2014). Induced resistance can result from various modifications in the plant: thickening of the parietal structures reinforcing their role of physical barrier, stimulation of secondary metabolic pathways allowing the synthesis of antimicrobial substances (such as phytoalexins) and accumulation of defense proteins (or PR proteins). This topic is currently the subject of a lot of research. To our knowledge, resistance to the mechanism of induced resistance has never been formally demonstrated, and due to the involvement of several defense compounds, it is considered unlikely to occur (Romanazzi et al., 2016). However, to survive and thrive in their hosts, plant pathogens have developed various strategies (Morrissey and Osbourn, 1999). These strategies include the excretion of toxic metabolites produced by the plants via efflux pumps, the degradation of defense compounds and the protection against oxidative damages (Bardin et al., 2015). These mechanisms of resistance to plant defense molecules might eventually lead to the selection of strains resistant to plant-induced resistance.

Finally, during the first stage of host-pathogen interaction, the synthesis of hydrolytic enzymes (cutinases, pectinases, etc.) by plant pathogens and, in particular, fungi is crucial for the infection process. Microbial bioprotectants can interfere with pathogenicity factors of the pathogens by inhibiting or degrading certain hydrolytic enzymes (Duffy et al., 2003). Additionally, some microorganisms can have an indirect effect on the establishment of pathogenesis, for example, by modifying the pH of the medium (Swadling and Jeffries, 1998). For example, an alkaline pH may reduce the efficiency of the enzymes necessary for a plant pathogen to degrade the tissues of the plants (Manteau et al., 2003). Microbial bioprotectants degrading oxalic acid, a molecule produced by

*Sclerotinia sclerotiorum* or *B. cinerea* during their interaction with the plant, can protect plants against attacks by these pathogens (Schoonbeek et al., 2007). Some bacteria have the ability to change the surface characteristics of plant leaves by synthesizing compounds with a surface-active effect, such as lipopeptides (Ongena and Jacques, 2008). This has the effect of hampering the process of attachment and growth of several plant pathogens on the leaves. In addition to this effect, cyclic lipopeptides are known to have a direct antimicrobial effect on plant pathogens, and they can elicit induced systemic resistance in certain host plants (Falardeau et al., 2013). According to several authors, the development of resistance to lipopeptides in microorganisms seems unlikely because of their biosurfactant properties which tend to disrupt or inhibit microbial biofilms (Zhao et al., 2017), and because their mode of action directly targets membranes, whose repair implicates a costly mechanism at the cellular level for microorganisms (Falardeau et al., 2013). However, very different levels of sensitivity to the lipopeptides Fengycin and Surfactin, both produced by *Bacillus subtilis*, were observed in two strains of *Venturia inaequalis* (Desmyttere et al., 2019). But to our knowledge, mechanisms of resistance to lipopeptides have not been described in plant pathogens.

These modes of action are not exclusive and a combination of mechanisms has been demonstrated for various bioprotectant microorganisms. The most studied case concerns the fungal genus *Trichoderma*, for which different modes of action have been demonstrated for the same strain (Elad, 2000; Harman, 2006; Howell, 2003; Karlsson et al., 2017), combining, for example, hyperparasitism and antibiosis (Lorito et al., 1993). According to available references, the strain QST713 of *Bacillus subtilis* acts by secretion of metabolites with antimicrobial activities including lipopeptides, by nutrient and spatial competition, and by induction of systemic plant resistance resulting in increased synthesis of peroxidases. This multitude of modes of action allows this bacterium to control various plant pathogens such as *B. cinerea*, *Pythium ultimum*, *Rhizoctonia solani*, *Plasmodiophora brassicae*, or the fungal agents responsible for powdery mildew (Lahlali et al., 2013; Levasseur et al., 2005; Paulitz and Belanger, 2001). In the case of the combination of several modes of action for a given microbial bioprotectant, the role and precise importance of each of them and the conditions under which the microorganisms switch from one mode of action to another in the control of the disease are not yet clear. The different modes of action may be expressed successively, simultaneously or synergistically, depending on the environmental conditions encountered.

The durability of biocontrol may depend on the mode of action involved in the effect of microbial bioprotectant on pathogens. All microbial bioprotectants can generate selection pressure on populations of plant pathogens when applied for crop treatments. However, certain modes of action are probably more conducive to the development of resistance than others. This crucial question will



have to be tackled by research in the future, in order to select modes of action with the most durable efficacy possible.

#### 4 Diversity of susceptibility of plant pathogens to microbial bioprotectants

The diversity in the level of susceptibility to microbial bioprotectants within plant pathogen populations may reduce their efficacy in the field. Due to the existence (even before the use of bioprotectants) of less sensitive phenotypes in natural populations of plant pathogens, the widespread use of bioprotectants could lead to rapid selection for resistance. Testing a sufficiently high number of isolates of a target plant pathogen for its level of resistance to a microbial bioprotectant is, therefore, the first step to estimate the risk of emergence of resistance. In addition, monitoring the level of resistance in natural populations of a plant pathogen before and after treatment can provide information on the distribution of resistance in the field, its impact on the efficacy of protection and its evolution over time.

A rapid assessment of resistance to fungicides in large collections of strains of fungal plant pathogens is commonly carried out by plant protection companies. This may be done, for example, according to the recommendations of the Fungicide Resistance Action Committee (FRAC), to establish a baseline for these products (<https://www.frac.info/>).

This type of study is increasingly carried out for microbial bioprotectants used against plant diseases. As shown in the review by Bardin et al. (Bardin et al., 2015), the diversity of the efficacy of microbial bioprotectants against plant pathogens has mainly been studied for microbial bioprotectants that produce metabolites with a direct effect on plant pathogens. Several studies have established a baseline for molecules produced by microbial bioprotectants. For instance, various levels of susceptibility to phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (2,4-DAPG), two compounds synthesized by antagonistic *Pseudomonads* present in the wheat rhizosphere, were detected among 66 strains of *Gaeumannomyces graminis* var. *tritici*, with one strain being resistant to both compounds (Mazzola et al., 1995). Various levels of susceptibility to 2,4-DAPG were also observed among 117 strains of *Fusarium oxysporum* (Schouten et al., 2004). A wide range of sensitivity to pyrrolnitrin, an antibiotic produced by microbial bioprotectants including *Pseudomonas chlororaphis*, was detected among 204 strains of *B. cinerea* (Ajouz et al., 2011b). Several studies have also been carried out on antibiotic-producing bioprotectants themselves. A wide spectrum of sensitivity to 19 antagonistic strains of *Streptomyces* sp. was, for example, identified among 15 strains of the plant

pathogenic bacterium *S. scabies*, the causal agent of potato scab (Otto-Hanson et al., 2013). Identically, an *in vitro* study revealed that 3 out of 41 strains of *B. cinerea* were not fully inhibited by any of the 3 antagonistic strains of *Streptomyces* sp. tested (Boukaew et al., 2017). The results of these two studies suggest the need to use a mixture of antagonistic *Streptomyces* sp. strains to effectively control the targeted diseases.

Scientific information available on the diversity of susceptibility of populations of plant pathogens to the protective effect of microbial bioprotectants that do not produce toxic metabolites or antibiotics against pathogens is scarce. However, available data show that, even in this case, a large diversity of susceptibility to microbial bioprotectants in populations of plant pathogens is generally observed. For example, in a study by Schnabel and Jones (Schnabel and Jones, 2001), considerable diversity in the susceptibility of 52 strains of *Erwinia amylovora*, the causal agent of fire blight, towards five specific bacteriophages was observed. According to Jones et al. (2012), bacterial strains within a species vary in their susceptibility to bacteriophages, suggesting that a mixture of bacteriophages is generally preferable for controlling bacterial strains present in the field. In order to ensure a durable efficacy of this method of protection, the authors recommend that a survey of resistance of bacterial strains should be carried out periodically by testing the sensitivity to virulent phages that have been deployed in the field (Jones et al., 2012). Another study has shown differences in susceptibility among 18 strains of *Erysiphe necator*, the causal agent of powdery mildew on grape, to the fungal hyperparasite *A. quisqualis* (Falk et al., 1995). Recently, the diversity for susceptibility to the fungus *Paraphaeosphaeria minitans* has been evaluated in the plant pathogenic fungus *S. sclerotiorum* (Nicot et al., 2019). This plant pathogen is responsible for white rot, which causes significant economic damage to a wide range of crops in most parts of the world (Bardin and Gullino, 2020; Boland and Hall, 1994). This fungus can attack more than 400 plant species, many of which are used in rotation cycles in arable cropping systems. In plant tissues contaminated with *S. sclerotiorum*, sclerotia, the resting structure of the fungus, are formed. Once incorporated in the soil of a field, the sclerotia constitute a stock of inoculum for the following years, and thus potentially contaminate subsequent crops. Sclerotia can survive for years in the soil (Bolton et al., 2006) and can produce either ascospores or mycelium, both of which can infect healthy plants at varying distances. Therefore, sclerotia constitute a source of primary inoculum playing a major role in the disease cycle. This disease is difficult to control by conventional methods, and currently, various microbial biocontrol products are approved worldwide (Bardin and Pugliese, 2020). The commercial bioprotectant Contans® contains as an ingredient the fungus *Paraphaeosphaeria minitans*, a hyperparasite that reduces the infectious potential of a field by parasitizing and destroying the sclerotia of *S. sclerotiorum*. Substantial variability in susceptibility to the commercial bioprotectant has been observed among 75 strains of *S. sclerotiorum*, regardless of

their geographic area and host of isolation (Nicot et al., 2019). This study suggests that the efficacy of the bioprotectant may vary from field to field depending on the presence of less sensitive strains. It also raises the question that selection pressure could possibly lead to a gradual selection of less susceptible strains, as this bioprotectant becomes widely adopted by farmers.

Other examples of diversity in susceptibility concern microbial bioprotectants combining several modes of action (Bardin et al., 2015). A recent study concerns the development of a new microbial bioprotectant adapted to tomato growing conditions in Algeria against *B. cinerea* that constitutes one of the major phytosanitary problems of greenhouse tomato crops in most parts of the world (Bardin and Gullino, 2020), including Algeria (Aissat et al., 2008). This disease is difficult to control by conventional control methods, and currently, several microbial biocontrol products are approved worldwide (Bardin and Pugliese, 2020; Nicot et al., 2016). However, the selection of new microbial bioprotectants is necessary in order to broaden the range of biocontrol tools for farmers and to offer solutions adapted to the climatic and cropping specificities encountered in certain countries of the world. To this end, a high potential microorganism, *Pseudomonas helmanticensis* CT22, has been selected to control grey mold in tomato greenhouses encountered in Northern Algeria (Bouaoud et al., 2018b). In order to evaluate the stability of efficacy of this potential new microbial bioprotectant towards *B. cinerea*, the susceptibility of various strains of the pathogen to two doses of the bioprotectant ( $10^7$  CFU/mL and  $10^6$  CFU/mL) has been tested (Bouaoud et al., 2018a). The efficacy of the bacterium was significantly influenced by the strains of *B. cinerea* tested at both doses of the product (ANOVA,  $P = 0.03$  and  $P < 0.001$  for high dose and low dose, respectively). At the 10-fold reduced concentration, a wide range of susceptibility was observed among the 62 strains of the pathogen tested with protection levels ranging from 24% to 100% (mean =  $80 \pm 3\%$ ; median = 87%; see Fig. 1). It reveals the importance of considering several strains of the pathogen when screening for new microbial bioprotectants, to obtain a good representation of the pathogen population and thus consider the future protective efficacy of the product in the field and its potential durability. The probable combined modes of action of this microbial bioprotectant (Bouaoud, unpublished data) and its high level of protection provided may reduce the risk of resistance development of *B. cinerea* strains.

Another example concerns the commercial product Serenade Max® (whose active ingredient is the bacterial strain *B. subtilis* QST713) that proved to have a significant protective efficacy against *B. cinerea* on various plant species including tomato (Bardin et al., 2013; Nicot et al., 2016). To assess the possible presence of low susceptibility to this microbial bioprotectant in populations of *B. cinerea*, the protective efficacy of this product was evaluated at two doses on whole tomato plants against 50

strains of the plant pathogen differing in their geographic area and host of isolation. At both concentrations of the product (low dose = 2 g/L and high dose = 8 g/L), a wide range of susceptibility was observed among the 50 strains, with protection levels significantly different according to the dose of product used ( $24 \pm 3\%$  at low dose and  $44 \pm 3\%$  at high dose, ANOVA,  $P < 0.001$ ). The efficacy of the product was significantly influenced by the strains of *B. cinerea* tested at both doses of the product (ANOVA,  $P < 0.001$ ), with some of the strains not controlled by the microbial bioprotectant (protection index less than 0) in the conditions of test on tomato plants (see Fig. 2).

There are other examples of diversity studies of susceptibility of plant pathogens to microbial bioprotectants (Bardin et al., 2015). All these studies clearly show that the protective efficacy of a microbial bioprotectant, whatever its mode of action, can vary depending on the strain of the plant pathogen. This observed diversity may contribute to the reduced efficacy of microbial bioprotectants in the field, by means of the existence of less susceptible phenotypes in natural plant pathogen populations and by means of the possible emergence of new resistant phenotypes from these less susceptible isolates due to the selection pressure exerted by the microbial bioprotectant used by farmers.

## 5 Risk of emergence of resistance to microbial bioprotectants in plant pathogens

An important issue concerns whether the widespread use of microbial bioprotectants in commercial fields or greenhouses could lead to the selection of resistant strains which may eventually jeopardize the efficacy of this control method, as it did in the case of chemical pesticide use. To elucidate this potential risk, repeated exposure of successive generations of a plant pathogen to compounds produced by a microbial bioprotectant or to a microbial bioprotectant itself needs to be realized. This procedure of experimental evolution is commonly carried out to evaluate the durability of the efficacy of antimicrobial agents in human pathology (Cowen, 2001) or to assess the capacity of fungal plant pathogens to adapt to fungicides (Brent and Hollomon, 1998). A first experiment conducted with the microbial bioprotectant *B. subtilis* CL27 in an *Astilbe hybrida* greenhouse revealed that after ten successive treatments the microbial bioprotectant had become ineffective against *B. cinerea* (Li and Leifert, 1994). Another documented example describes a procedure of experimental evolution done *in vitro* to test the hypothesis of an adaptation of a plant pathogen to the effect of a microbial bioprotectant in the presence of continuous selection pressure (Ajouz et al., 2010). This study reveals that *B. cinerea* was able to adapt to the effect of pyrrolnitrin, an antibiotic synthesized by several

microbial bioprotectants. Additionally, cross-resistance was observed between pyrrolnitrin and iprodione, a commonly used anti-*Botrytis* fungicide (Fillinger et al., 2012). It suggests a potential risk of development of resistance to this antibiotic and to the pyrrolnitrin-producing microbial bioprotectants in the field, even in the absence of treatment by the microbial bioprotectant itself. However, the fitness cost associated with this resistance was significant for the pathogenic fungus (Ajouz et al., 2011a). Thus, mutants of *B. cinerea* resistant to pyrrolnitrin showed reduced mycelial growth, a low level of aggressiveness on the host plant (tomato and apple) and a reduction in the capacity to produce conidia compared to sensitive wild-strains. This, therefore, suggests a limited capacity for epidemic dissemination of strains of *B. cinerea* resistant to this antibiotic.

Knowledge concerning the potential erosion of efficacy of microbial bioprotectants is essential to ensure a durable use of these products on target plant pathogens. This knowledge will help to identify risk factors that can foster the selection of resistant strains of plant pathogens to microbial bioprotectants. It will also help identify the types of microbial bioprotectants with a lower risk of loss of efficacy.

## 6 [Future trends in research and recommendations for a durable use of microbial bioprotectants](#)

The durability of the efficacy of microbial bioprotectants may be linked to the characteristics of plant pathogen populations, such as genetic diversity and the ability to evolve in response to selection pressure. The selection pressure exerted by field use of microbial bioprotectant depends on the surfaces treated, the application rates or treatment cadences. The outcome of this pressure for specific microorganisms ultimately depends on evolutionary mechanisms such as mutation, recombination, migration and selection. These mechanisms have been the subject of numerous studies for microorganisms in other contexts, for example, to assess the durability of plant varietal resistance to plant diseases. Thus, McDonald and Linde (McDonald and Linde, 2002) postulated that populations of pathogens with high evolutionary potential were more likely to overcome varietal resistance. The same hypothesis can be proposed for the development of resistance of plant pathogens to microbial bioprotectants. Although many plant pathogens are known for their capacity to develop resistance to conventional plant protection methods, only a few studies have explored their ability for resistance to microbial bioprotectants. Available studies highlight differences in susceptibility of various strains of plant pathogens to microbial bioprotectants or to compounds that are produced or synthesized as the result of their interaction with the host plant.

The durability of the efficacy of biocontrol may also be linked to intrinsic characteristics of the microbial bioprotectant such as its mode of action. Significant research efforts are, therefore, still necessary to acquire precise knowledge on the modes of action of microbial bioprotectants. This knowledge will make it possible to optimize their use and anticipate the potential failures of efficacy, for example, by recommending the use of bioprotectants combining several modes of action or of complex formulations (mixtures of metabolites or of microorganisms with different modes of action) or alternating products. It will also help to integrate durability concerns in the screening procedure for new microbial bioprotectants, aiming at the selection of the more durable modes of action and avoiding modes of action that can easily lead to the selection of resistant isolates in natural populations of plant pathogens.

If the commercialization of microbial bioprotectants became widespread in the world and their use increased in cropping systems (expansion of the treated surfaces, increase of the applied dose or in the frequency of applications), the emergence of new phenotypes resistant to these products could not be excluded. Data are still too scarce to allow the development of specific principles on necessary precautions for the use of microbial bioprotectants in practice. However, this chapter underlines the need to develop now the appropriate management schemes for these new products, in order to avoid repeating errors made in the past when deploying chemical fungicides or varietal resistance. One possible strategy could be to start applying for the deployment of commercial microbial bioprotectants some of the FRAC recommendations (<https://www.frac.info/>). Such recommendations may, for instance, concern the alternation of products with known differences in modes of action, the establishment of sensitivity baselines and the survey of evolution in populations of the plant pathogens and the development of risk prediction models. Another strategy can be suggested for the selection of new microbial bioprotectants, such as the use of more than one strain of the target plant pathogen during the screening procedure, the use of criteria to maximize durability (e.g. avoid certain modes of action, privilege combined modes of action) and promote the development of mixtures of strains (assembled consortia) with complementary modes of action. However, on this last point, regulatory, technical and cost issues may complicate the commercialization of assembled consortia.

## 7 Where to look for further information

The following articles provide a good overview of key aspects of the subject:

- Bardin, M., Ajouz, S., Comby, M., Lopez-Ferber, M., Graillet, B., Siegwart, M., and Nicot, P. C. (2015). Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? *Frontiers in Plant Science* 6, 566.
- Bardin M, Nicot P. 2020. Chapter 11. Using microorganisms as biocontrol products. In : Fauvergue et al. eds. *Biocontrôle. Eléments pour une protection agroécologique des cultures* (p. 151-160). Quæ. (in French)
- Bardin, M. & Pugliese, M. (2020), Biocontrol agents against diseases. In: Gullino, M., Albajes, R. & Nicot, P. (eds.) *Integrated Pest and Disease Management in Greenhouse Crops*. Springer.
- Bardin M, Siegwart M. 2020. Chapter 23. Can pests develop resistance to biocontrol products? In : Fauvergue et al. eds. *Biocontrôle. Eléments pour une protection agroécologique des cultures* (p. 299-305). Quæ. (in French)
- Köhl, J., Kolnaar, R. & Ravensberg, W. J. (2019), Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. *Frontiers in Plant Science*, 10, 845. doi: 10.3389/fpls.2019.00845
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- van Lenteren, J. C., Bolckmans, K., Köhl, J., Ravensberg, W. J., and Urbaneja, A. (2018). Biological control using invertebrates and microorganisms: plenty of new opportunities. *BioControl* 63(1), 39–59

Research in this area is conducted by member institutions of the International Organization of Biological Control (<https://www.iobc-global.org/>) and in particular by scientists participating in the activities of the following Working Groups of the West Palaearctic Regional Section:

- Biological and integrated control of Plant Pathogens ([https://www.iobc-wprs.org/expert\\_groups/06\\_wg\\_plant\\_pathogens.html](https://www.iobc-wprs.org/expert_groups/06_wg_plant_pathogens.html)).
- Induced resistance in plants against insects and diseases ([https://www.iobc-wprs.org/expert\\_groups/17\\_wg\\_induced\\_resistance.html](https://www.iobc-wprs.org/expert_groups/17_wg_induced_resistance.html)).
- Integrated control in Protected crops, Temperate climate ([https://www.iobc-wprs.org/expert\\_groups/08\\_wg\\_protected\\_crops\\_temperate.html](https://www.iobc-wprs.org/expert_groups/08_wg_protected_crops_temperate.html)) and Mediterranean climate ([https://www.iobc-wprs.org/expert\\_groups/09\\_wg\\_protected\\_crops\\_mediterranean.html](https://www.iobc-wprs.org/expert_groups/09_wg_protected_crops_mediterranean.html)).

- Integrated control in Citrus fruit crops ([https://www.iobc-wprs.org/expert\\_groups/16\\_wg\\_citrus\\_crops.html](https://www.iobc-wprs.org/expert_groups/16_wg_citrus_crops.html)).
- Integrated protection of Field vegetable Crops ([https://www.iobc-wprs.org/expert\\_groups/12\\_wg\\_vegetables.html](https://www.iobc-wprs.org/expert_groups/12_wg_vegetables.html)).
- Integrated protection of Fruit crops ([https://www.iobc-wprs.org/expert\\_groups/02\\_wg\\_fruit\\_crops.html](https://www.iobc-wprs.org/expert_groups/02_wg_fruit_crops.html)).
- Integrated protection of Olive crops ([https://www.iobc-wprs.org/expert\\_groups/15\\_wg\\_olive\\_crops.html](https://www.iobc-wprs.org/expert_groups/15_wg_olive_crops.html)).

Recommendations and strategy suggested for the deployment of chemical fungicides in Agriculture can be found online at the Fungicide Resistance Action Committee: <https://www.frac.info/>

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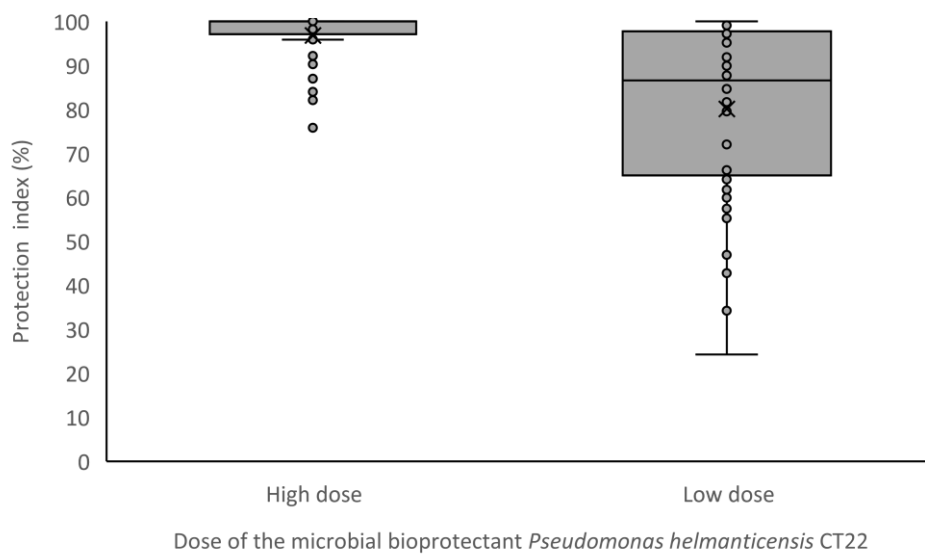


Figure 1 Box plot representing the diversity of protection triggered by the microbial bioprotectant *Pseudomonas helmanticensis* at high dose ( $10^7$  CFU/mL) and low dose ( $10^6$  CFU/mL) against 62 strains of *Botrytis cinerea* on tomato plants. The protection index was computed as  $100 * (AUDPC_{BC} - AUDPC_{CT22}) / AUDPC_{BC}$ , where  $AUDPC_{BC}$  was the average AUDPC for a given strain of *Botrytis cinerea* and  $AUDPC_{CT22}$  was the average AUDPC for the same strain in presence of the microbial bioprotectant. For each boxplot, per cent of protection for each strain (○), mean (x), median (horizontal line), 25–75% values (grey box), and maximum and minimum values (vertical line) are represented.

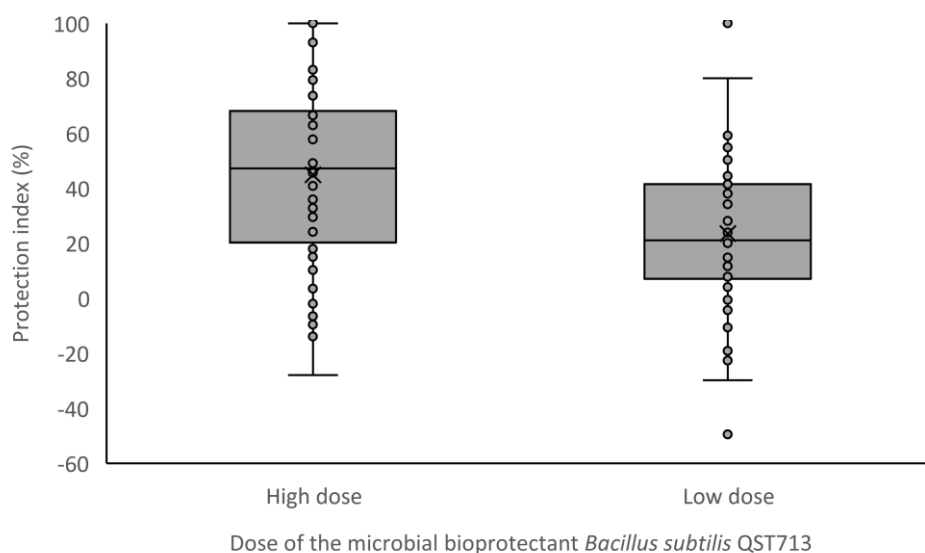


Figure 2 Box plot representing the diversity of protection triggered by the microbial bioprotectant *Bacillus subtilis* QST713 at high dose (8 g/L) and low dose (2 g/L) of the commercial product Serenade Max<sup>®</sup> against 50 strains of *Botrytis cinerea* on whole tomato plants. The protection index was computed as  $100 * (AUDPC_{BC} - AUDPC_{ser}) / AUDPC_{BC}$ , where  $AUDPC_{BC}$  was the average AUDPC for a given strain of *Botrytis cinerea* and  $AUDPC_{ser}$  was the average AUDPC for the same strain in presence of the microbial bioprotectant. For each boxplot, per cent of protection for each strain (○), mean (x), median (horizontal line), 25–75% values (grey box), and maximum and minimum values (vertical line) are represented.