Control of soilborne pathogens of tomato using a commercial formulation of Streptomyces griseoviridis and solarization

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Improving the efficacy of biocontrol agents against soilborne pathogens[1]

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Abstract

Technical, economical and environmental factors are forcing to adopt new sustainable methods, such as the use of microbial antagonists, for the control of soilborne pathogens. Research has mainly focused on antagonistic fungi and bacteria, often not providing consistent or satisfying results. Biocontrol agents can be combined with other chemical products, with physical methods (solarization or steam sterilization) or agronomical practices, such as enhancement of suppressive soils, use of amendments or microbial optimization in the case of soilless systems. Different biocontrol strategies should be developed for different pathogens. The use of microorganisms can play an important role in a more complex vision of crop protection, as a key elements of IPM programs. Mixtures of antagonists with complementary activities could be developed, but the need for multiple registration is critical. Genetic manipulation could result in new biocontrol strains with increased production of toxic compounds or lytic enzymes, improved space or nutrient competence, wider host range or enhanced tolerance to abiotic stresses. The potential risks related to the environmental release of genetically modified microorganisms must be carefully assessed. Genes

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and enzymes involved in the biocontrol mechanism could be applied directly or transferred to crops. Finally, the production system, formulation and methods of application (soil and seed treatment or microbial colonization of the hydroponic nutrient solution) are crucial to maintain and improve the efficacy of microbial antagonists.

Keywords: antagonist, biological control, formulation, integrated control, genetically modified microorganism, mass production, method of application, risk assessment, soilborne diseases.

1. Introduction

Meeting the goal of improving soilborne disease control for efficient and sustainable production systems requires a reduction of the chemical inputs in agriculture. Various movements or parties see increased pesticide application as a threat to public health and the environment. Agronomists experience growing problems with the build up of resistance to the pesticides in target pathogen populations (Dekker, 1976; Brent and Hollomon, 1998) and the withdrawal of pesticides from the market (Gullino and Kuijpers, 1994). Of special relevance, in the case of many vegetable and ornamental crops, is the loss of methyl bromide as soil fumigant (Katan, 1999; Gullino et al., 2003). Furthermore, the private industry is coming across increasing costs in discovering and developing new molecules for disease control application. Consequently, there is a growing awareness that integrated pest management (IPM) strategies can provide more environmentally sound and economically feasible alternatives for soilborne disease control. Biological control with introduced microorganisms has been identified as an important component of IPM strategies (Cook, 1993).

Many studies have been devoted to the identification of microorganisms able to reduce the activity of soilborne pathogens during the four past decades (Baker and Snyder, 1965).

Many laboratories around the world have developed their own microorganisms and this allowed the collection of important contributions about the biology of pathogens and antagonists. However, in

spite of the initial great optimism and extensive later research efforts, progress in achieving commercial, large-scale usage of biological pest control has been slow.

Most experiences were on a laboratory scale, thus avoiding the problems related to antagonist mass production and biofungicide formulation, and disease control trials were performed in simplified environments such as growth chambers, experimental greenhouses or small field plots, thus avoiding the risk of large scale experiments. When trials move towards the farm scale, many antagonists fail to confirm their activity or, when tested in different environments, behave unpredictably, and consequently lack reliability (Mathre et al., 1999).

Biofungicides presently meet many constraints and it is not a straightforward task to find, develop and implement practically feasible biocontrol products against soilborne diseases. Nevertheless, there are also reasons for stating careful optimism. There are many possibilities for combining various biocontrol agents, with each other, or with agronomical, physical or chemical control methods. In particular, by combining different methods of control, the aim is to obtain a synergistic effect, rather than additive. For that reason, a complete comprehension of the mode of action and mechanism of control is needed. A good combination is obtained when both partners benefit from the combined treatment. For example, combining a biocontrol agent (BCA) with a fungicide improves the control by the BCA and, at the same time, it enables to reduce the fungicide dosage, rendering its use more acceptable. Moreover, the combination of control methods provides a wider spectrum of control, which is especially needed to replace the wide spectrum fumigants, such as methyl bromide. Finally, combining different control methods enables to make use of less effective control methods which cannot stand by themselves, but in combination they can contribute to the control.

Other possibilities to improve the antagonist effectiveness include the genetic manipulation of the microorganisms and the enhancement of the mass production, formulation and methods of application The main possibilities to improve the efficacy of the biocontrol agents against soilborne pathogens are the topic of this brief review.

2. Combination with chemical pesticides

The combined use of BCAs (biocontrol agents) and chemical pesticides has attracted much attention in order to obtain synergistic or additive effects against the target organisms (Locke et al., 1985). Reduced amounts of fungicide can stress and weaken the pathogen and render its propagules more susceptible to subsequent attack by the antagonist (Hjeljord and Tronsmo, 1998). Replacement of some of the chemical fungicide treatments with BCAs does not only reduce the input of chemicals but can also result in improved disease control. For this reason, BCAs are required to be resistant to chemicals. Either the natural resistance of some antagonists towards specific fungicides could be exploited or resistant mutants can be produced, by selection on pesticide-containing media (Abd-El Moity et al., 1982), by UV-induced mutation (Postma and Luttikholt, 1993), or by protoplast fusion (Minucci et al., 1991).

The high antagonistic activity of benzimidazole resistant isolates of *Fusarium* antagonists permitted the combination of chemical and biological control measures, leading to improved control of fusarium wilt of carnation or cyclamen (Garibaldi et al., 1990a; Minuto et al., 1995a). Seed treatments with BCAs which are insensitive to pesticides used in traditional seed technology are used in commercial delivery systems based on antagonistic *Trichoderma* spp. (Harman and Björkman, 1998). Some heavy metal-resistant mutants of *Trichoderma* spp. selected on heavy metal-rich artificial media were effective antagonists of *Fusarium* spp., *Pythium* spp. and *Rhizoctonia* spp. (Kredics et al., 2001). These mutants might be of value for use with heavy metal-containing pesticides, as part of an integrated plant protection system.

By using chemical and biological control measures together, the duration of active disease control will be extended and the chances for the development of fungicide resistance can be reduced. Chemicals are effective under climatic conditions or level of disease pressure in which the antagonist is less effective, but a BCA can colonize roots, wounds or senescing plants tissue before the pathogen (Ahmad and Baker, 1987; Garibaldi et al. 1990b; Hjeljord and Tronsmo, 1998).

3. Combination with soil disinfestation

Soil disinfestation is commonly used for sanitation in low technology houses and fumigation with chemicals, heating, steaming and solarization are the main methods in use (Garibaldi and Gullino, 1995). Soilborne pathogens were effectively controlled by using methyl bromide (Bell et al., 1996). However, concern regarding the potential of methyl bromide to deplete ozone led to its inclusion among the substances controlled by the Montreal protocol which use must be eliminated by the end of 2004 from industrialized countries (Gullino et al., 2003). Although the targets are soilborne pathogens, soil disinfestation can also have deleterious effects on beneficial soil microorganisms, but some genera, such as *Trichoderma* spp. and *Gliocladium* spp., are often less sensitive to fumigants and other chemicals used in disinfestation, resulting in recolonization of the soil by these organisms (Garibaldi and Gullino, 1995). There is, however, a high risk of recolonization of the biological vacuum, with pathogens, leading to more serious disease problems (Jarvis, 1989). Incorporation of antagonists following disinfestation of the soil has been shown to be effective in controlling *Rhizoctonia solani* on carrots (Strashnow et al., 1985), *Rh. solani* and *Sclerotium rolfsii* on tomato and peanuts (Elad et al., 1982). Resting propagules of pathogens such as microsclerotia can be predisposed to infection by *Gliocladium* spp. as a result of the treatment (Tjamos, 1992).

Among available alternatives, biological control and soil solarization, alone or in combination, are promising methods for soilborne disease control (Minuto et al., 1995b; Katan, 1996). Solarization, carried out by covering the soil with transparent film during the hot season, has been widely exploited in a number of warm countries as well as in climatically marginal ones (Katan and DeVay, 1991). In Northern Italy, its efficacy is improved when applied in the greenhouse (Garibaldi and Gullino, 1991). Solarization proved to be a potential control strategy both in combination with *Trichoderma harzianum* against *Rhizoctonia solani* (Chet et al., 1982), as in combination with *Gliocladium virens* against southern blight of tomatoes (Ristaino et al., 1991). Sivan and Chet (1993) combined the application of *Trichoderma harzianum* with soil solarization under field

conditions, obtaining a significant disease control of fusarium crown and root rot of tomato and a significant yield increase. Minuto et al. (2004) demonstrated that the combination of soil solarization and *S. griseoviridis* was effective against fusarium and verticillium wilts and corky root, increasing the range of pathogens controlled with respect to the single treatments. A significant increase in the weight of the single fruit was noticed and a higher yield per square meter, when solarization and biocontrol agents were applied together, confirming the potential additive effect caused by the commercial biofungicide and solarization in terms of yield increase. Combining BCA with solarization may also enable the shortening of the solarization process and the use under marginal conditions.

BCAs can also be applied after steam disinfestation of soils and substrates, an approach largely applicable in the case of ornamental and vegetable crops (Hoitink et al., 1991; Gullino, 1992**).**

4. Application of agronomical practices for biocontrol

Several agronomical methods permit to promote biocontrol agents. These include advantageous regimes for choosing crop plants, soil types, crop rotations, soil amendments, and the application of suitable ploughing, planting and sowing regimes.

Natural disease suppressive soils are good examples in which the indigenous microflora effectively protects plants against soilborne pathogens. Suppressive soils have been described for many soilborne pathogens, including *Fusarium oxysporum* (Scher and Baker, 1982; Alabouvette, 1986; Garibaldi et al., 1990b), *Gaeumannomyces graminis* var. *tritici* (Shipton, 1975), *Pythium ultimum* (Garibaldi et al., 1993), and *Rhizoctonia solani* (Lucas et al., 1993). Suppressiveness can be induced, transferring the characteristics to conducive soils. Isolation of microorganisms responsible for suppressiveness has permitted to inoculate soils and substrates, seeds and multiplicative material with the antagonists (Garibaldi et al. 1987). Some biofungicides have been developed studying suppressive soils. A biofungicide (White et al., 1990) based on the K61 strain of *Streptomyces griseoviridis* isolated from *Sphagnum* peat (Tahvonen, 1982) can control or suppress some root rot

and wilt diseases, caused by *Pythium* spp., *Fusarium* spp., *Rhizoctonia* spp. and *Phytophthora* spp., colonizing the rhizosphere prior to pathogens. A strain of *Gliocladium virens* isolated in the late 1980s from a soil in Maryland, was developed to control *Pythium ultimum* and *Rhizoctonia solani* in soilless mixes (Lumsden and Locke, 1989). The nonpathogenic *Fusarium oxysporum* strain Fo47, originally isolated from a fusarium suppressive soil in France is marketed as liquid formulation for soilless culture such as tomato in rockwool (Alabouvette et al., 1998). Another strain of *F. oxysporum* isolated from soils suppressive to fusarium wilt of carnation is the active ingredient of a biological product against different *formae speciales* of *F. oxysporum* (Aloi et al., 1992).

Organic amendments such as green manures, stable manures, and composts have long been recognized to facilitate biological control if applied before planting (Lumsden et al., 1983). A great diversity of microorganisms contribute to biocontrol provided by composts (Kwok et al., 1987; Alvarez et al., 1995). Fluorescent pseudomonads *Pantoea* and *Bacillus* spp. were the most effective species against Pythium damping-off on cucumber (Boehm et al., 1997) present in a composted pine bark-amended potting mix. Moreover, composts can serve as an ideal source of nutrients for antagonists into soils and offer an opportunity to introduce and establish specific BCAs into soils, which in turn leads to a sustainable disease control based on the activities of microbial communities (Hoitink and Bohem, 1999). At least two separate mechanisms of biocontrol exist in suppressive composts. The first, typical of properly prepared composts, is general suppressiveness that operates primarily against *Pythium* and *Phytophthora*, based on the competition of nutrients. If composts lack readily-available nutrients, these pathogens may be suppressed (Hoitink et al., 1991). The second mechanism of suppression is more specific, it is required for pathogens such as *Rhizoctonia solani* and it appears to be related primarily to mycoparasitism (Hoitink et al., 1997). Strains of *Trichoderma hamatum* and *Flavobacterium balustinum* are among the most effective strains (Kwok et al., 1987).

Greenhouse conditions offer good opportunities for the application of BCAs, mostly due to their more uniform and controlled environment (Albajes et al., 1999). Antagonist to be used in greenhouses should be selected in order to fit the set of conditions, such as pH, temperature and matrix potential, of the cultivation environment (Paulitz and Bélanger, 2001). Moreover, cultural practices and amendments inducing disease suppressiveness in soil or soilless media are more effective in greenhouses than in the open field.

In closed recirculating soilless systems, zoosporic pathogens can easily spread in the water systems, in absence of the microbial buffer produced in soil by the activity of the saprophytic microflora (Gullino and Garibaldi, 1994). Fortunately, under such conditions it is quite easy to introduce a BCA at the beginning of the cropping period to prevent colonization of the substrate by the pathogenic fungi. *Trichoderma* spp. are naturally found as a part of the indigenous microflora in soilless systems (Jensen and Lumsden, 1999). Also bacteria seem to be well adapted to soilless cultivation due to the high water content of such systems (Paulitz, 1997). Some strains of *Pseudomonas fluorescens* and *P. corrugata* reduced zoospore germination and motility of *Pythium* sp. rot in hydroponically cultivated cucumber (Rankin and Paulitz, 1994). A good example of exploitation of biocontrol in hydroponic cropping is represented by the microbial optimization of soilless systems through the application of microorganisms able to colonize the rooting system of plants grown under a strictly controlled environment (Van Os and Postma, 2000). A slow sand filtration technique was tested both alone and in combination with different antagonistic strains belonging to *Fusarium* spp. and *Trichoderma* spp., isolated from gerbera rhizosphere and applied into the soilless systems (Grasso et al., 2003). This disinfection technique can be successfully combined with the application of antagonistic microorganisms (Garibaldi et al., 2003).

5. Mixtures of antagonists

Association of several microorganisms is needed to control different diseases that affect the same crop. Most of the BCAs are specific only for a given type of target pathogen. Although this property

represents an advantage from the environmental point of view, it creates great difficulties to the growers who need to control several plant pathogens in the same crop. Moreover, the combination of two or more antagonists means also multiple registration processes, with increased costs and difficulties in matching all the studies required by the strict legislation. However this option could be feasible with products already registered: biofungicides based on different antagonistic strains may be labeled as compatible with each other and proposed for joint use.

Alabouvette et al. (1996) demonstrated that a synergistic effect can be obtained in controlling *Fusarium oxysporum* f.sp. *radicis-lycopersici* by combining a fluorescent *Pseudomonas* sp. with a non-pathogenic *F.oxysporum*. The non-pathogenic *F.oxysporum* competes for carbon sources while the bacterial antagonist produces a siderophore competing for iron (Lemenceau et al., 1993). Moreover it was noted that the antagonistic strain Fo47 was less sensitive to pseudobactin-mediated iron competition than the pathogenic *F. oxysporum* f.sp. *dianthi*. Park et al. (1988) also showed that interaction between the bacterium *P. putida* and saprophytic strains of *F.oxysporum* could achieve effective control of *Fusarium oxysporum* f. sp. *cucumerinum*.

A positive, possibly synergistic, interaction between *Trichoderma* spp. strains and bacterial antagonists, such as *Pseudomonas syringae* has been reported for combined applications in the control of plant pathogens (Whipps et al., 1997). Part of the mechanism could be explained with the positive interaction among the lipodepsipeptides of the bacterial antagonist and the fungal cell wall-degrading enzymes of the fungal biocontrol agent (Fogliano et al., 2002). An effective control was also demonstrated for the combination of *Trichoderma harzianum* protecting against infection by *Pythium ultimum* in the rhizosphere and *Pythium nuun* reducing inoculum density of the same pathogen in the soil mass (Paulitz et al., 1990).

By combining specific strains of microorganisms, multiple traits antagonizing the pathogen can be combined and this may result in a higher level of protection. When *P. putida* strain WCS358, competing for iron through the production of its pseudobactin siderophore, was combined with *P. putida* strain RE8, inducing systemic resistance against *F. oxysporum* f.sp. *raphani*, fusarium wilt

255 suppression was significantly enhanced (de Boer et al., 2003). Previously, a mixture of three
256 different plant growth-promoting rhizobacteria (PGRP), applied as a seed treatment, showed
257 intensive plant growth promotion and reduction of multiple cucumber diseases (Raupach and
258 Kloepper, 1998). Another innovative approach for improving soilborne disease control could be the
259 development of cocktails containing strains that communicate with each other to maximize
260 antibiotic production and disease control (Becker et al., 1997; Davelos et al., 2004).
261 Hoitink et al. (1991) incorporated several antagonists in combination in peat substrates rendering
262 them disease suppressive. A broad spectrum biological control of *Pythium*, *Phytophthora*, and
263 *Rhizoctonia solani* requires the introduction into or presence of organic nutrients in the soil to
264 maintain several taxa of biocontrol agents (Hoitink and Boehm, 1999). The composition of the
265 microflora active in control changes as the organic matter decomposes, while the microbial carrying
266 capacity of the amendment declines.
267
268 **6. Genetic manipulation**
269 Despite years of research and development, significant questions regarding the physiological and
270 ecological constraints that limit biological control remain unanswered. Molecular and genomic tools
271 offer new possibilities for improving the selection, characterization, and management of biological
272 control. Development in proteomics and functional genomics will give us the possibility to
273 determine and follow expression of crucial genes of BCA's during mass production, formulation,
274 storage and application. Moreover, molecular techniques allow modification of wild type strains to
275 improve their ability to control soilborne diseases. Unwanted genes might be deleted, or one or
276 more genes, coming from different antagonists, could be added.
277 For fungi without functional or easily induced sexual stages, four methods are usually employed for
278 genetic manipulation: conventional mutagenesis induced by chemicals (Howell and Stipanovic,
279 1983) or by ultraviolet light (Baker, 1989); protoplast fusion (Harman et al., 1989; Minucci et al.,
280 1991); transposon mutagenesis (Brown and Holden, 1998); and transformation. Most frequently,

281 protoplasts are used for introduction of exogenous DNA in presence of polyethylene glycol (PEG)
282 and calcium chloride (Penttilä et al., 1987). Whole fungal cells can be transformed using the lithium
283 acetate method (Dickman, 1988), by electroporation (Goldman et al., 1990), by particle
284 bombardment (Lorito et al., 1993) or by Agrobacterium-mediated transformation (Zeilinger, 2004).
285 Bacteria transformation can be performed by using electroporation, osmotic shock, or *Escherichia*
286 *coli*-mediated conjugation.
287 One of the modes of action of antagonists is mycoparasitism, due to the production of lytic
288 enzymes, such as chitinases, proteases and glucanases, able to degrade the pathogen cell walls.
289 Genes encoding chitinases have been cloned from a wide variety of microorganisms, including
290 *Serratia marcescens* (Sundheim et al., 1988) and *Trichoderma harzianum* (de la Cruz et al., 1992:
291 cloning of *chit33*; Garcia et al., 1994: cloning of *ech42*). Baek et al. (1999) reported an increased
292 and decreased antagonistic activity against *Rhizoctonia solani* on cotton strains of *T. virens*
293 containing two *ech42* copies and a disrupted *ech42* gene copy. With constitutive expression of
294 *chit33* in *T. harzianum*, the recombinant strains provided a superior biocontrol activity against *Rh.*
295 *solani* on agar plates (Limon et al., 1999). An endophytic strain of *Pseudomonas fluorescens* has
296 been transformed with the *chiA* gene encoding the major chitinase of the *S. marcescens*, showing an
297 effective control of *Rh. solani* on bean seedlings under plant growth chamber conditions (Downing
298 and Thomson, 2000).
299 Chitinase genes may also be used to improve resistance of plants against fungal pathogens. Genes
300 from biocontrol fungi encoding endochitinases or exochitinases were inserted into potato and
301 tobacco (Lorito et al., 1998), apple (Bolar et al., 2001), broccoli (Mora and Earle, 2001), or
302 *Brassica juncea* (Mondal et al., 2003).
303 Many bacterial or fungal strains used in biocontrol produce antibiotics that inhibit the growth of
304 other fungi. Introduction of the gene encoding the housekeeping sigma factor into a strain of
305 *Pseudomonas fluorescens* increased the production of pyoluteorin and 2,4-diacetylphluoroglucinol
306 (DAPG) (Schnider et al., 1995). Another strain of *P. fluorescens*, antagonist of *Rh. solani* in

seedlings, has been genetically modified for increased production of the antibiotic pyrrolnitrin (Ligon et al., 1996), by modifying or introducing an extra copy of the wild type global regulator gene *gacA*. The GacS/GacA two-component system globally exerts a positive effect at a posttranscriptional level on the production of extracellular metabolites required for the control of plant diseases in many pseudomonads (Haas and Keel, 2003). A strain of *Pseudomonas putida* has been genetically modified to have improved activity against soil-borne pathogens, carrying the *phz* or the *phl* biosynthetic gene loci and constitutively producing either phenazine-1-carboxylic acid (PCA) or DAPG (Bakker et al., 2002). Genes for the biosynthesis of antibiotics can be transferred to antagonistic strains extending the range of pathogens controlled. Transfer of the *phzH* gene of *P. chloraphis*, necessary for the biosynthesis of phenazine-1-carboxamide (PCN) and the control of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, to the PCA-producing biocontrol strains *P. fluorescens* and *P. aureofaciens* enabled these strains to produce PCN instead of PCA and suppress tomato foot and root rot (Chin-A-Woeng et al., 2001).

Soil bacteria, called plant growth promoting-rhizobacteria (PGPR), are found to be beneficial to the plant. Indole-3-acetic acid (IAA) production by PGPR may play a role in the construction of short root systems and giving advantage of root colonization (Suzuki et al., 2003). The molecular mechanisms involved in the root colonization are under study nowadays. The *rpoS* gene in *Pseudomonas putida* was essential for plant root colonization under competitive conditions from other microbes (Miller et al., 2001). Camacho Carvajal et al. (2002) have characterized a NADH dehydrogenases of *Pseudomonas fluorescens* WCS365 and their role in competitive root colonization. These PGPR could be genetically modified to improve root colonization and, consequently, biocontrol of phytopathogens. The introduction of the *sss* colonization gene of the tomato-*Fusarium oxysporum* f. sp. *radicis-lycopersici* biocontrol strain *Pseudomonas fluorescens* into poor and good colonizer strains of *P. fluorescens* increased the competitive tomato root tip colonization ability several times, showing that improvement of the colonization ability by genetic engineering is a realistic goal (Dekkers et al., 2000). Moreover, avirulent mutants of *Ralstonia*

333 *solanacearum* have been used in the biocontrol of bacterial wilt in potato (Smith and Sadler, 2001).
334 These strains containing transposon-induced insertions in the *hrp* gene (Frey et al., 1994) were able
335 to invade the plant, survive and multiply within the plant excluding pathogenic strains.
336 The role of siderophore production by biocontrol agents in the rhizosphere has been studied with
337 molecular methods for many years (Haas, 2003). A strain of *Pseudomonas fluorescens* was
338 genetically modified to utilize additional ferric siderophores (Moenne Loccoz et al., 1996).
339 A new and challenging branch of genetic transformation of biocontrol agents could be the insertion
340 of genes that improves tolerance of antagonist to abiotic stresses, such as genes for increased
341 tolerance or resistance to cold, heat, drought, high salinity, heavy metal rich soils, or acid soils.
342 Although dependent on political decisions, if genetically modified antagonists could be used
343 unrestrictedly, various efficient combinations of plant pest resistance and biocontrol regimes could
344 then be designed. A possible crop protection method could then be the creation of plant cultivars
345 promoting survival and activity of specific BCAs.
346 In order to release GM-biocontrol agents into the environment – at this stage there are great
347 difficulties in registering GM-BCA – a careful evaluation of the potential risks associated should be
348 carried out. Safety issues involve displacement of nontarget microorganisms, allergenicity or
349 toxicity to humans and other animals, toxicity and pathogenicity of nontarget organisms, genetic
350 and transgene stability (Cook et al., 1996; van Elsas and Migheli, 1999). In particular, genetic
351 stability of both manipulated and wild-type bacteria and fungi is a key factor in their safe
352 application. Care should be taken to ensure maximum stability of the transforming DNA within the
353 host genome and to avoid horizontal gene transfer. The strain K1026 of *Agrobacterium radiobacter*,
354 derived from the strain K84 producer of agrocin 84 and effective antagonist of *A. tumefaciens*, was
355 designed with a deletion in the transfer region of the plasmid (Jones et al., 1988). The resulting
356 strain is unable to transfer its genes giving immunity to agrocin 84. If new transgenic hypervirulent
357 biocontrol agents are created, the prevention, containment, or mitigation of uncontrollable spread of
358 such microorganisms becomes a urgent need. Gressel (2001) proposes a series of potential means,

359 such as asporogenic deletion mutants, transgenic suppression of melanization, terminator strategy or
360 hypervirulence genes flanked with transgenetic mitigators.
361 Finally, the members of the APS Biological Control Committee asserted that significant investment
362 in genomic analyses of biological control agents will lead to increased efficacy and application of
363 biological control of plant pathogens and recently proposed a list of recommended BCAs for
364 genomic sequencing.
365
366 **7. Mass production**
367 A critical factor that must be considered when selecting a BCA for commercial development is the
368 availability of a cost-effective production and stabilization technology that yields an optimally
369 effective form of the antagonist. More studies on the practical aspects of mass-production and
370 formulation need to be undertaken to make new biocontrol products stable, effective, safer and
371 more cost-effective (Fravel et al., 1999).
372 Major characteristics to market a biofungicide are the following (Agosin and Aguilera, 1998):
373 abundant and cost-effective production of microbial propagules; ability to survive downstream
374 process; stability and adequate shelf life of the final product upon storage, preferably without
375 refrigeration; tolerance to environmental variations in temperature, desiccation, irradiation and
376 relative humidity in order to survive and establish active populations in the soil; and consistent
377 efficacy under varying field conditions at commercially feasible rates.
378 One of the most important reasons for the limited commercial diffusion of biofungicides is the high
379 cost of production, due to the high cost of substrate, low biomass productivity, and limited
380 economies of scale (Rhodes, 1996). The practical efficacy of a BCA greatly depends on the quality
381 of the inoculant, itself a function of the production and formulation processes (Whipps, 1997).
382 Two methods are commonly used for producing inoculum of BCA's: liquid and solid fermentation.
383 In submerged fermentation, specific parameters for aeration, temperature and pH control, carbon
384 and nitrogen sources must be developed for each organism. Extra-cellular chitinase production by

385 the chitinolytic fungus *Trichoderma harzianum* using submerged fermentation was studied
386 (Sandhya et al., 2004). Supplementation of additional carbon sources showed no further
387 enhancement in chitinase production while supplementation of nitrogen sources such as peptone
388 and tryptone in the fermentation medium showed a marked increase in production. Many BCAs are
389 easily produced in liquid culture in lab scale, but when produced in large scale, they do not produce
390 the expected quantity or quality of propagules, essentially for the low oxygen availability in
391 fermenters. Some filamentous fungi need high oxygen transfer for growth and especially for
392 sporulation.
393 The choice of the adequate propagule of the microorganism is important to provide the desired shelf
394 life. Some bacteria are easily dried and can be provided as dry cells or formulated further. Survival
395 stage structures of the organism, such as chlamydospores, microsclerotia, ascospores, or endospores
396 are generally preferred. Sporulating Gram-positive bacteria, such as *Bacillus* spp. and *Streptomyces*
397 spp., offer endospores resistant to desiccation and heat that can be formulated readily into stable
398 products, such as dry powder. Instead, Gram-negative bacteria, such as Pseudomonas spp., are
399 generally formulated as frozen cell pellets that must be kept at low temperature until application,
400 which remain a major obstacle for their large-scale use (Slininger et al., 1996).
401 If the microorganism is produced by liquid fermentation, it is necessary to reduce the volume of
402 liquid or obtain a final dry formulation. Before drying, the microorganism propagules are separated
403 by filtration or centrifugation. The strain Fo47 of *Fusarium oxysporum* has been produced in
404 submerged fermentation, removing the growth medium by filtration and the propagules were mixed
405 with talcum powder used as an inert carrier and then dried at 18-20°C for two days (Durand et al.,
406 1989). Drying can also be accomplished by freeze-drying, atomization, or bed-fluid drying,
407 preserving the inoculum for a long time with high viability (Beudeker et al., 1989). Rapid drying
408 can cause cell membrane damage, particularly if heating is used to speed drying. For this reason, a
409 glycerol-enriched medium was developed to produce high levels of desiccation-tolerant conidia of
410 *Trichoderma harzianum* (Jin et al., 1996).

A biofungicide based on the K61 strain of *Streptomyces griseoviridis* is produced by fermentation followed by lyophilization. The commercial product contains at least 10^8 dormant spores/g and is stable for 12 months at temperatures inferior to 8°C (Tahvonen and Avikainen, 1987). Sarabatnam and Traquair (2002) demonstrated that vegetative propagules from actively growing filaments are acceptable inoculants for *Streptomyces* sp. formulations with better shelf life at 4°C. The final product is easy to apply by mixing it with nutrient solution delivered to plants in soilless cultures or by mixing with potting mixtures.

Solid-state fermentation mimics the natural environment conditions and habitat for some microorganisms (Kim et al., 1985). The moisture needed is found in the solid matrix in an adsorbed or complex form, with moisture ranging between 12 and 80%. Solid fermentation scaling, necessary for use on an industrial scale, raises engineering problems due to the build-up of temperature, pH, O_2, substrate and moisture gradients. However solid fermentation possess several biotechnological advantages, among the other, higher fermentation productivity, higher end-concentration of products, higher product stability, and lower demand on sterility due to the low water activity (Holker et al., 2004). Larena et al. (2002) produced 250-fold more conidia of *Penicillium oxalicum*, antagonist of *F.oxysporum* f.sp. *lycopersici*, in solid than in liquid fermentation. Conidia produced in solid fermentation had a longer shelf life if stored at –20°C.

Moreover, solid-state fermentation permits to save the labor and technical difficulties and generally does not need further formulation. This type of formulation is adapted for horticultural usage where it is mixed with potting mixture, but it does not enable application of the inoculant as a suspension in water. The strain Fo47 of *F. oxysporum* can be produced by solid-state fermentation either in sterilized peat or in calcinated clay, and be stored at 4°C or at room temperature without loss of density or activity (Olivain et al., 1999). Conidia of *Trichoderma harzianum* produced in aerial mycelium with solid-substrate fermentation persist longer under harsher environmental conditions thanthose produced under submerged culture conditions and wall thickness of aerial conidia is nearly twice that of submerged ones (Muños et al., 1995).

A new solid-state fermenter patented by Lüth and Eiben (2003) was used for *Coniothyrium minitans* spore production. A big advantage was the culture of the fungus under absolute axenic conditions. After 14 days of sterile fermentation - including *in situ* drying - the cereal grains used as a substrate were covered and filled with fungal spores. When the fermentation process is completed the conidia are separated from the culture medium using a micro-screen machine, that permits to have only the conidia of the processed fungus in the end product.

8. Formulation and methods of application

Biological control has often been idealized as method for controlling plant diseases, as has happened for organic farming compared to the traditional cropping techniques. In organic farming the bottleneck is represented by keeping remunerative yield, lowering the selling price of the product. For biological control the major difficulty to reach the market and to be competitive with the chemical fungicides is represented by a consistent and reliable effectiveness and by the length of shelf life. Both problems can be faced with a scientific development of formulation of biocontrol agents. Until now, except for rare cases, formulation has been faced with an empirical approach without a methodology.

Obvious advantages of formulation include greater efficacy, increased shelf life, ease of handling, increased safety, lower production costs and compatibility with agricultural practices. Minuto et al. (1997) have compared different strains of antagonistic *Fusarium oxysporum* and different commercial formulations of these strains to control fusarium wilt of basil, concluding that the efficacy was strongly dependent on the formulation.

Formulation is dependent on the type of fermentation used. In the case of solid-state fermentation providing the carrier for the inoculum, it is not necessary to develop a sophisticated formulation process (Lewis, 1991).

The type of formulation desired depends on the intended use. For application to soilless cultures where the easiest way is to apply the inoculant through the drip irrigation system, a liquid

formulation would be preferred. A granular material would be more appropriate for combining with potting mix, while a wettable powder would be more appropriate for root dips or sprays. The application of *Coniothyrium minitans* follows one of two ways: either soil application to reduce the sclerotial inoculum-potential, or spore-sprays onto diseased plants or crop debris to sanitize the crop (de Vrije et al., 2001).

Often a biofungicide comprises many ingredients, such as carriers, diluents, bulking additives, membrane stabilizers, growth and contaminant suppressants, buffering systems, binders, dispersants, lubrificants, activators, food sources and coating compounds, added for various purposes (Paau, 1998). These include keeping viability of antagonists, manipulating bulk for handling and delivery, promoting the activity of the BCAs, and arresting growth of potential contaminants.

More attention should also be devoted to the special requirements of BCAs in terms of application and delivery technology. Delivery must be easy, effective, timely, to the appropriate site of action, and compatible with available agricultural equipment. In contrast to chemical pesticides, they are living organisms, they range in size and are more susceptible to the actual conditions (temperature, humidity, pH) than chemical pesticides (Matthews, 2000). In general, most biocontrol agents are applied with the same equipment currently used to apply chemical pesticides (Mathre et al., 1999).

9. Conclusions

The history of biological control of plant pathogens is not so recent. Sandford in 1926 started biocontrol against potato scab, but research on this topic received a renewed impetus and attracted many scientists after the 1963 International Symposium held at Berkeley on Ecology of soilborne plant pathogens: prelude to biological control (Baker and Snyder, 1965; Baker and Cook, 1974). Despite many decades of research in biological control, biopesticides represent about 1% of the global pesticide market (Butt et al., 2001). BCAs still represent a specialized niche market, in spite

of a consensus view among the public, growers and regulators. BCAs are generally characterized by a narrow spectrum of activity, which is desirable from an environmental point of view, but not from a commercialization perspective. International agrochemical companies, characterized by large sales forces and substantial fixed costs, are not generally interested in scarcely remunerative niche products. Small enterprises, the portion of the production system normally interested in developing such a products, should have long term planning, because return from the initial costs is quite long. Biofungicides have to break into the existing agro-industrial complex: they might act differently than chemical pesticides in traditional testing situations largely built for chemical fungicides and they might need certain special arrangements for their application (Gerhardson, 2002).

Regulatory guidelines for biocontrol agents (BCAs) in many parts of the world are incompletely formulated or subject to frequent changes. Moreover, not all countries adopt a favorable and fast registration process for BCAs: in Europe as in the United States the small enterprises, which would develop products based on BCAs, should be prepared to deal with the complex procedure and high costs for registration of microbials (Hofstein et al., 1996; Harman, 2000).

Despite the difficulties encountered by the development of biofungicides, there are many reasons for being optimistic. Different strategies can be established to speed up the production, commercialization and acceptance of biocontrol agents and effectiveness of control. The use of microorganisms can play an important role in a more complex vision of crop protection, as key elements of IPM programs. Mixtures of antagonists, genetically modified antagonists, enhancement of production, formulation, and application technologies and procedures will be useful tools to accomplish this difficult and exciting task.

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