CHAPTER 13

AN INTEGRATED APPROACH TO BIOLOGICAL CONTROL OF PLANT DISEASES AND WEEDS IN EUROPE

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1. Introduction

Biocontrol has been ineffective against major agricultural pests in the field, and has not provided the tools to cost-effectively compete with chemical pesticides, despite the theoretical benefits. The ecological and evolutionary reasons for the lack of effectiveness have been examined in detail in a recent book (Vurro *et al.,* 2001a) along with suggestions on how to safely enhance their activity.

The advent of the first call of the 6th European Union[a] Framework Programme for Research and Technological Development requesting research projects looking for "safer and environmentally friendly production methods and technologies and healthier foodstuffs", with the specific challenge "to develop lower input farming systems based on systems such as integrated production and organic agriculture", provided the impetus to found a consortium to enhance biocontrol agents so that they might actually fill the gap.

Suitable targets were chosen and a team organized with the necessary expertise. Plans were advanced with reasonable objectives and the scientific activities integrated, and the project was funded. This integrated project is described below.

2. The targets: uncontrollable agricultural pests

Among all the living organisms that can attack crops causing qualitative and quantitative reduction of production, those living in the soil, such as plant pathogens and weeds are among the worst and the more difficult to control by traditional methods and strategies.

2.1 Soil borne plant pathogens

Soil borne plant pathogens are a major problem in many open field and greenhouse crops. Pathogens are often able to survive for several years in the soil as dormant, environmentally persistent resting structures, until a susceptible crop is introduced. The pathogens responsible for damping off, crown and root rots, as well as wilts are of utmost importance in vegetable crops. Various *Pythium, Rhizoctonia,* and *Phytophthora* spp. Damage the lower part of tomato, pepper, cucumber, and many other vegetables, both in soil and soil-less cultures. *Sclerotinia sclerotiorum* is an important soil-borne pathogen responsible for the rot disease of over 400 plant species, including economically important field and glasshouse crops (Boland & Hall, 1994), and survives between crops in the soil as sclerotia (Coley-Smith & Cooke, 1971;

J. Eilenberg and H.M.T. Hokkanen (eds.),An Ecological and Societal Approach to Biological Control, 257–274.

Merriman, 1976). These sclerotia may germinate, producing mycelia that then infect the plant directly or, more typically in glasshouse crops, produce large numbers of ascospores with the potential to infect plants over a wide area. The crown-rot and wilt-inducing strains of *Fusarium oxysporum* are responsible for severe damage to many important crops (tomato, cucumber, muskmelon, asparagus, radish, onion, flax, carnation, and cyclamen). *Fusarium* wilt pathogens have a high level of host specificity and are classified into more than 1200 *formae speciales* and races.

2.2. Parasitic and perennial weeds

The parasitic *Orobanche* species (broomrapes) attack nearly all vegetables, legumes, and sunflowers in southern Europe to the Balkans and Russia, the Middle East and North Africa. *O. ramosa* and *O. aegyptiaca* infest about 2.6 millions hectares planted in the Solanaceae and grain legume crops, particularly tobacco, potato, tomato, eggplant, chickpeas, peas, and faba beans (Sauerborn, 1991). *O. cumana* severely restricts and limits sunflower production in Spain and eastern Europe. The broomrapes interfere with water and mineral intake and by affecting photosynthate partitioning, and are responsible for both qualitative and quantitative damage to these high value crops.

These parasitic plants can produce 10^4 - 10^5 seeds per flower stalk, which can remain viable for many years. They germinate after stimulation by host root exudates, and produce a germ tube that can attach and develop a haustorium penetrating the root, forming a tubercle. This is followed by the most damaging phase, with the parasitic withdrawal of water, nutrients and photosynthates from the host. Due to the long underground tubercle phase, flower stalk emergence occurs only when most of the damage has already been produced.

Perennial weeds are among the most troublesome weeds to manage. *Cirsium arvense* is considered one of the world's worst weeds (Holm *et al*., 1977), and the third most important weed in Europe (Schroeder *et al*., 1993). The weediness of this species is largely attributed to its capacity for vegetative reproduction and regenerative growth by recruitment of shoots from adventitious buds on a creeping root system (Donald, 1994). *Sonchus arvensis* is another perennial species that presents a considerable weed control challenge, especially to organic farming. *Cyperus esculentus*, another top ten "Worlds Worst Weeds" (Holm 1977) is one of the most serious invasive alien species in southern Europe, both in crops and non crop areas.

3. Traditional solutions: usefulness and limits

For years, the most common approach to the control of the above pest problems was soil fumigation, before or after cropping. Many fumigants are health hazards, environmental pollutants, and even contribute to atmospheric ozone depletion. Increased environmental concern has triggered regulatory restrictions on treatments with soil fumigants, drastically reducing methyl bromide use, which had been the most widespread and effective soil fumigant. Fumigants such as 1,2-dibromochloropropane (DBCP) and ethylene dibromide (EDB) are generally less effective than methyl bromide in controlling pathogens and weeds. Their use has been discontinued or suspended in many countries. In many crops no real alternatives to methyl bromide have been found. Furthermore, soil fumigants (as well as solar heat treatment) reach pathogens and weed seeds in all physical and biological niches in the soil. As a result, their use often also leads to the eradication of beneficial organisms, and a negative shift in the biological equilibrium. This creates a biological vacuum, which then leads to an increase in the population of pathogens, causing more damage than those originally targeted for control. Soils, especially those with low microbial populations are more vulnerable to reinvasion of pathogens following fumigation. Thus, non-chemical methods of selectively controlling soilborne diseases and weeds are needed.

Other control strategies such as soil solarization (allowable in organic agriculture) could be possible, but have environmental and temporal constraints, as warmer temperatures than normally found in Europe are needed and the fields must remain covered for much of a growing season. Seed treatments with conventional fungicides provide some initial protection to soil pathogens, but this is not effective for a sufficient duration in heavily infested soils. None of the old and environmentally unfriendly fungicides still allowable in organic agriculture (copper salts, sulfur) are very effective against soilborne pathogens.

Traditional control methods have been tried against parasitic weeds on different crops, but none have proved to be effective. *Orobanche* spp. usually cannot be managed by persistent selective herbicides, since herbicides are not able to differentiate between the crop and the parasite, except with herbicide-resistant transgenic crops (Joel *et al*., 1995; Aviv *et al*., 2002) or with mutant crops such as imidazolinone resistant sunflowers. Multiple applications of low rates of crop-degraded herbicides can provide a modicum of control and may be more useful when integrated with other methods, such as biological control (Hershenhorn *et al*., 1998). Furthermore, as these weeds attach to crop roots, they cannot be controlled mechanically, except by removing their flower stalks to reduce seed accumulation and dispersal. Recently, inexpensive seed treatments have been developed for a different parasitic weed – *Striga* in Africa, using 20 times less herbicide than would be sprayed on a field, yet providing season long control (Kanampiu *et al*., 2003).

Perennial weeds are difficult to control using traditional methods because they usually cannot be easily removed mechanically, due to their well developed root systems or subterranean organs, and because repetitive chemical treatments are often required, which are expensive in conventional agriculture. None of the few very old and environmentally unfriendly herbicides (sulfuric acid, perchlorate, soaps) allowable in organic agriculture actually control perennial or parasitic in weeds. Weed control is considered the major expense in, and major biotic limitation to, organic agriculture. Soil degrading and energy expensive mechanical cultivation as well as back-breading manual weeding are the major alternatives to herbicides, but are also ineffectual against perennial and parasitic weeds, suggesting the need for new paradigms to deal with an old problem.

4. Biological control with fungi: a solution with benefits and limits

Some effort during the last few decades has been dedicated to biological control of weeds and plant diseases, but it is a fraction of the efforts expended on developing new chemical pesticides.

Many potential microorganisms were found, but their use is still very limited. This is due to many evolutionary constraints, including: biological (virulence, stability, defence mechanisms of the target pest, interaction with other microorganisms); technological (poor sporulation, lost of aggressiveness, special growth requirements); environmental (need for extended dew periods for establishment, physical characteristics of the soil (physical and chemical barriers) and commercial (limited market, registration problems including secondary toxicity and registration costs, costs of production).

All the host-specific organisms proposed for inundative biocontrol had evolved to be in equilibrium with their weed/pathogen hosts. The hypervirulence needed by the farmer could lead to mutual extinction of the biocontrol agent and its host. Thus, virulence of the organisms must be enhanced to overcome these evolutionary barriers to provide similar disease and weed control as conventional pesticides. Only a limited number of commercial products are available against a few of the greenhouses diseases, and no commercial bioherbicides are available in Europe. Cost has not been the major limiting factor for the adoption of biocontrol agents for the pest constraints discussed above. If effective, almost anything could compete with the cost of methyl bromide in high input agriculture, or with mechanical cultivation in low input agriculture. The major problems have been with consistency and lack of near complete control activity, when biocontrol agents are active. The systems used by the team are described below.

4.1. Coniothyrium minitans

Coniothyrium minitans is an obligate mycoparasite of ascomycetous sclerotium-forming fungi, including important plant pathogenic species of *Sclerotinia* and *Sclerotium,* such as *Sclerotinia sclerotiorum*, *S. minor*, *S. trifoliorum* and *Sclerotium cepivorum* (Whipps & Gerlagh, 1992). They act by infecting and reducing the viability of the sclerotia in soil.

Unlike other mycoparasites of sclerotia, such as certain *Trichoderma* species and *Sporidesmium sclerotivorum, Coniothyrium* does not grow through soil and initially would not appear to be a likely candidate for long-term successful biocontrol. Nevertheless, it survives well in soil, and can be recovered three years after application in the field (McQuilken *et al*., 1995). Other mechanisms of its spread are involved, such as water splash and aerosol dispersal, which allow sclerotia of *S. sclerotiorum* to be infected over 2 metres away from a source of *C. minitans*. It is also dispersed by slugs, collembolans, mites and sciarid larvae (Turner & Tribe, 1976; Williams *et al*., 1998 a, b; Whipps, 1993; Whipps & Budge, 1993).

The potential use of *C. minitans* as a biocontrol agent by soil incorporation of solid substrate has long been recognised. The organism has been successfully used in glasshouse and field experiments to control *Sclerotinia* diseases of a number of crop plants (Whipps & Lumsden, 2001). A commercial product has been registered in seven European countries, the USA, and Mexico. The major constraints of its wider use in agricultural practice in the field outside of glasshouses are the limited knowledge of its ecology, and the scanty information on its physiology and genetics, preventing attempts at strain improvement.

4.2. Trichoderma spp.

Trichoderma strains are among the more effective fungi applied against fungal diseases, both in conventional and organic farming. They are commercially produced and several patents protect their use (Harman et al., 1994, 1996). Biopesticides based on antagonistic *Trichoderma* strains are used for biocontrol of phytopathogenic fungi causing root and crown rot of vegetable seedlings damping off, vascular diseases, ‘take all’ of cereals, etc. (Harman & Björkman, 1998). *Trichoderma* strains are registered both in Europe and USA as the active principles of biopesticide formulations, and are allowed in organic farming.

Regardless of the obvious potential, there are some problems that limit the development and application of these biofungicides. In addition to the lack of strains for every disease, and of very effective and optimally-formulated preparations, there is a limited availability of basic information for further product registration, including a sufficient knowledge of the mechanisms of action and interaction with other biocontrol agents. More efficacy tests are needed in each geographic area where the product has to be registered. Methods for monitoring the production of possibly mammalian toxic metabolites produced by some of these fungi are necessary to allow an evaluation of possible risks derived from large scale application.

4.3. *Antagonistic Fusarium spp.*

The concept of using non-pathogenic strains of *Fusarium oxysporum* to control *Fusarium* diseases came from the demonstration that the suppression of the disease in suppressive soils results from interactions between pathogenic and non-pathogenic strains. Therefore, non-pathogenic strains were developed as biocontrol agents (Lemanceau & Alabouvette, 1991). The non-pathogenic *F. oxysporum* strains have several modes of action contributing to their biocontrol capacity (Couteaudier & Alabouvette, 1990; Lemanceau *et al*., 1993). They are able to compete for nutrients in the soil, suppressing pathogen chlamydospore germination. They can also compete for infection sites on the root, and can trigger plant defence reactions, inducing systemic resistance (Fuchs *et al*., 1997). Several strains of non-pathogenic *F. oxysporum* have good efficacy in many trials, but as with other biocontrol agents, there is a lack of consistency.

4.4. Potential mycoherbicides

Despite isolation of many promising pathogenic organisms that could be useful for control of parasitic weeds, none has received continual widespread use. Two very promising strains of *F. arthrosporioides* and *F. oxysporum* were isolated in Israel from juvenile *O. aegyptiaca* plants. They also attacked *O. ramosa* and *O. cernua* (Amsellem *et al.*, 2001). Some very promising strains were also isolated in Italy, (Boari & Vurro, 2004). These species can be formulated as mycelia, reducing the dew period and the expense of spore production (Amsellem *et al.,* 1999). It was also possible to enhance their activity two fold by engineering in genes for overproduction of auxin (Cohen *et al*., 2002), but more than a doubling of virulence is needed.

Perennial weeds in arable farming are ideal targets for biological control, that could replace one or more herbicide treatments. In organic farming systems, biological control of perennials, especially *Cirsium arvense*, would reduce the number of time consuming, expensive, and soil degrading mechanical treatments that require large amounts of fossil fuel compared to biocontrol and to herbicides.

Phomopsis cirsii, Ramularia circii, and *Septoria cirsii* were chosen as promising candidates in systematic field surveys of diseased *C. arvense* carried out in Denmark (Leth & Andreasen, 1999) and Russia (Berestetski, 1997). Their necrotrophic nature makes them able to grow in liquid artificial media. *Sonchus arvensis* is another perennial species that is an ideal target for biological control. Several virulent pathogens have been isolated by the partners (Berestetski & Smolyaninoca, 1998), but their efficacy has to be fully evaluated and improved.

5. The team

The group has been gathered on the basis of the proven excellence of each partner in a field of research and its ability of carrying out innovative activities in biological control. Among the nine partners, coming from seven different countries (as shown in the map), there had been complementarities and interactivity through international projects and COST actions (http://cost.cordis.lu). Many team members participated in a workshop on enrich biocontrol agents (Vurro *et al.*, 2001a). Each group has a long tradition in research on biological control, leading to important scientific, technological and applicable contributions in their respective complementary fields of interest.

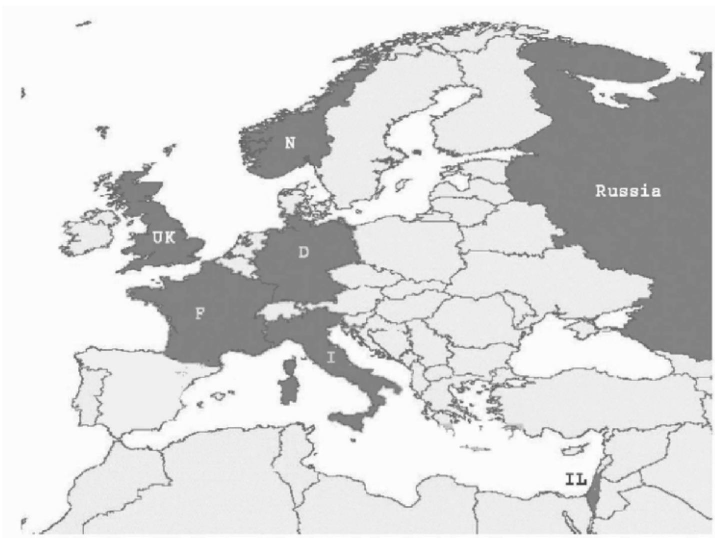

Figure 1: European countries involved in the project (in dark grey)

Many different microorganisms are considered into the project, and thus, many different types of biotechnological, molecular, physiological, and applicative expertise were needed. Even though each group works on the organisms on which it has already accumulated a high level of knowledge, this expertise will be made available for the enhancement of other's microorganisms. Each partner works in collaboration with several partners, in more than one task, and on more than one organism. For example, four partners are involved with *Coniothyrium* studies, five on *Trichoderma*, four on antagonistic *Fusarium*, six on perennial or parasitic plants.

Each working group includes experts in mycology, physiology, biotechnologies, molecular biology, chemistry, weed and crop science, allowing for multifaceted work-plans. Each partner has well suited laboratories for the project activities. A continuous flow and exchange of materials, strains, technologies and protocols has been created within sub-packages, which should allow attaining the planned objectives.

6. The project

The project has been divided in nine interactive and transversal work-packages, starting from the genetic and physiological enhancement of microorganisms to their application and the assessment of field efficacy, until the evaluation of food quality after their use, and the acceptability by consumers. Some of the main tasks of each work-package are briefly illustrated below.

6.1. Efficacy enhancement through the knowledge of genetic characters

Scientific risk assessment is used throughout the project to ascertain where risk may be of consequence, as well as to inform the public and allay not always rational fears, where appropriate, or to devise methods to minimize risk when there is one.

Within the project, the extensive use of genetic tools and techniques has the main aim to understand the modes of action of biocontrol agents and to manipulate gene expression to enable their better and safe use in the future. Thus, transgenics are considered to suppress the production of mammalian toxins by otherwise excellent "natural" biocontrol agents. A further important aim is to study new instruments to obtain more efficacious fungal strains, and to carefully ascertain the impact of their release.

One of the activities planned is to identify changes in enzyme production and gene expression by biocontrol agents during infection of the host. The spectrum of enzymes involved in biocontrol activity is known to include glucanases, chitinases, lipases and proteases but knowledge of their quality, regulation and characterisation is often poor (Lorito *et al*., 1993). Knowledge of these factors should help deployment of biocontrol agents under optimal environmental conditions for activity. Even though the involvement of cell-wall degrading enzymes in pathogenicity by fungal biocontrol agents is well established, the changes in genes controlling other pathogenicity traits are not well known. The project examines changes in gene expression during early stages of infection. Genes differentially expressed during various stages of infection will be identified and cloned using macro and microarray technologies and suppressive subtractive hybridisations using genomic and stage specific libraries (Yang *et al*., 1999). Transgenic overexpression of these same genes can later be considered to enhance virulence.

It is essential to identify the biocontrol agent following application and during ecological impact and risk assessment studies concerning the use of biocontrol agents in the glasshouse and field. One way of doing this is to introduce genetic markers that facilitate easy recovery and monitoring, and such genetically-marked strains of *Coniothyrium minitans* and *Fusarium oxysporum* are already available (Eparvier & Alabouvette, 1994) for use in the environmental impact studies. Nevertheless, other DNA based marker systems such as "biobarcoding" with pre-planned and easy to detect nonsense (non-coding) sequences (Gressel & Ehrlich, 2002) may prove valuable, potentially more sensitive and environmentally safer alternatives to those containing coding genetic inserts.

Several transformation-based techniques are beginning to appear to allow reproducible genetic modifications in fungi. It should be possible to knock out genes in the biocontrol agent as well as to transfer specific genes into the biocontrol agent, and then determine effects on pathogenicity. This will demonstrate the role of any gene in pathogenicity and, in the long term, will enable the development of more effective biocontrol agents.

The characterisation and utilisation of mating type genes in biocontrol strains to improve mycoherbicide activity is another objective. *Fusarium oxysporum* and several other *Fusarium* species that reproduce asexually harbour mating type genes, which were appropriately transcribed and processed (Yun *et al*., 2000). The presence of mating type genes in asexual species of *Fusarium* and the fact that they are fully functional (Moretti *et al*., 2002) is consistent with the hypothesis that asexual fungi may have a cryptic sexual cycle, even though sexual structures have never been identified in these fungal species. This will allow recognition of compatible strains that can be used in crossing experiments within each species to obtain sexual states. *In vitro* crosses to assess their sexual compatibility will be made based on this information. Strains will be selected for crossing that have high levels of pathogenicity and toxicity, to increase the chances of obtaining strains with higher levels of both traits among the progeny.

Fusarium spp. that have been engineered to control *Orobanche* spp. with genes for the over-production of auxins, provided a modicum of increased virulence, but only when the fungi were preloaded with tryptophan, a precursor for IAA biosynthesis (Cohen *et al*., 2002). Similarly a *Colletotrichum* sp. controlling *Abutilon* transformed with the same genes, was exceedingly hypervirulent, when the fungus was sprayed together with tryptophan. Mutants of many species have been selected for overproduction of anthranilate synthase by using tryptophan analogs as selectors, overproduce tryptophan (Romero *et al*., 1995), and may be useful here.

The production of asporogenic mutants of biocontrol agents would allow propagation and preclude off-target movement, as well as prevent their environmental persistence (Gressel, 2001; 2004). An important part of the task will be the study of hypervirulent and safe mycoherbicides.

6.2. Physiological enhancement of biocontrol activity

Different approaches are being used to increase the efficacy of biocontrol agents without using genetic or transgenic manipulation. New protocols will be tested with different species of biocontrol agents, including both mycoparasites and mycoherbicides, permitting the development of novel and fully integrated protocols to simultaneously enhance pathogen and weed control.

Molecular activation of specific genes occurs during the antagonist-pathogen and antagonist-plant interactions. The production of inducers of mycoparasitism released from the pathogen or the plant and "detected" by a fungal biocontrol agent has been recently demonstrated (Lorito *et al*., 2001). The project plans to identify and characterize both proteins and small molecules (as well as the genes specifically induced) produced during the complex interaction between antagonistic fungi, the plant and the pathogenic fungi, as well as in the presence of symbionts. The molecules identified will be tested for their capacity to induce physiological alterations in the plant that correlate with resistance (i.e. production of PR-proteins, accumulation of salicylic acid, accumulation of Ca^{2+}, oxidative burst and increased resistance to foliar pathogens) and control the antagonist-pathogen-plant interaction to improve biocontrol by *Trichoderma*.

The transgenically enhanced hypervirulence of a biocontrol agent has the advantage of constitutiveness: it is there, and there are no needs for additives. Conversely, if the same effect can be achieved physiologically by an additive, then there is the advantage that the organism is no different from the wild type after the additive has dissipated. For example, organisms can be mutated to supply tryptophan to an organism with the potential to be hypervirulent via over production of auxin. Conversely, an organism could be engineered to overproduce oxalate (Gressel, 2002), to overcome calcium dependent weed defenses, or the biocontrol organism could be provided with exogenous oxalate to achieve the same hypervirulence (Gressel et al., 2002), yet the organism lacks hypervirulence when the oxalate is gone.

6.3. Ecological fitness

The biocontrol agents used in this project are expected to control soil-borne diseases or weeds, and therefore they will be directly or indirectly applied to soil. To be efficacious, biocontrol agents must establish, survive and be active in soil. Physical and chemical characteristics in soil influence the population dynamics of microorganisms. Factors such as the proportion of sand and clays, the nature of the clays, the organic matter content and the pH are very important in relation to survival and activity of microorganisms introduced in soil (Alabouvette & Steinberg, 1995; Höper *et al*., 1995). The ecological fitness (Butt *et al*., 2001) of the biocontrol agents selected during this project will be assessed, studying the ecological behaviour of the biocontrol agents in relation to soil type, climatic conditions, temperature and water potential, as well as crop species to be protected.

6.4. Environmental impact of biocontrol agents

The environmental impact of a variety of biocontrol agents will be assessed by tracking their movement, assaying non-target effects and any changes in host range (especially after genetic or physiological modifications), together with determining long term environmental persistence. All these together are part of risk assessment to evaluate whether risk mitigation is needed, developing the tools for this, where necessary.

A major effort is devoted to the identification of molecular markers to recognize strains of biocontrol agent after their release into the soil. The genetic diversity within species will be determined by using DNA molecular analysis such as sequencing of the nuclear ribosomal DNA, beta-tubulin gene, calmodulin gene and elongation factor gene, and AFLP. The sequence data obtained along with comparing sequence data available in the EMBL/GenBank databases, will allow screening for the determination of probes that would lead to the development of species-specific discriminatory primers. The AFLP polymorphisms will be used as tools for obtaining markers of fungal populations and to detect polymorphisms between the target and non-target species, to provide maximal flexibility for subsequent primer design. A real-time PCR assay will be set up using the primers designed for the detection of the biocontrol agents, as it provides both qualitative detection and quantitative determination of the fungal pathogen eliminating post-PCR processing.

The introduction of biocontrol agents into soil may pose a risk of unforeseen or detrimental activities on the soil microbial population. The EU directive 2001/36 clearly says that side effects on non target soil microorganisms should be addressed, but there are no validated methods available. Until recently, techniques for monitoring direct effects on microorganisms have been restricted to *in vitro* culture based methods that ignored 90% or more of the microbial population that could not grow on culture media in the laboratory. The study of the composition of the microbial communities will be based on the direct extraction of DNA from soils. Improvements or developments will be required to address the diversity of fungi and protozoa communities. Terminal restriction fragment length polymorphism analysis (T-RFLP) and ribosomal intergenic spacer analysis (RISA) will be adapted to the analysis of soil communities. The molecular markers revealing shifts in the structure of the microbial communities will be cloned and sequenced for a subsequent comparison with data sets available in international databases. This will allow the identification of the microbial groups appearing as putative bio indicators of the transient and longer-term impact of biocontrol agents.

Biocontrol fungal strains, such as *Fusarium oxysporum* and *F. arthrosporioides,* transformed with innocuous markers *GUS* and *GFP* genes together with a hygromycin resistance gene are already available to follow their “natural” movement and persistence in the field. An alternative way to allow simple recognition of competing organisms in the same habitat is the insertion of non-coding biobarcode™ sequences (Gressel & Ehrlich, 2002) having universal primer pairs and variable generated sequences, using algorithms a group member developed for identifying organisms. The use of the biobarcode concept would require regulatory acquiescence that non-coding sequences are not genes, and thus the organisms bearing such sequences are not “genetically modified” in the legal sense.

6.5. Cost effective production of competitive biocontrol agents

Biological control of plant diseases, insect pests, and weeds will only successfully compete with chemical pesticides if the products are as effective as the chemical products and if they are not more expensive and complicated to use. Apart from the efficacy of the strain of the microorganism used, this is mainly dependent on how it was produced and formulated. The production technology used must ensure the highest possible yield of live propagules. The formulation must ensure an application of the propagules to the soil or to the plant, as easy or nearly as easy as the application of a chemical pesticide. One paradigm for development of biocontrol agents states that the formulation must improve or at least assist the effectiveness of the microorganism and must ensure a shelf life of the product of at least one year, better two or more years. A different paradigm states that the only element is cost-effectiveness that is competitive with current technologies. In these days of inventory control, shelf life is less important, and if the product is really good, yet requires special application techniques, custom applicators and farmers will invest in the equipment, just as they had for specific equipment for methyl bromide fumigation.

Suitable culture media for the production of the fungal propagules will be selected using an appropriate fermentation technology followed by: the evaluation of the most suitable growth conditions; the selection of the best technology to separate the propagules from the fermentation product; the evaluation of the most suitable methods and conditions for the formulation of the propagules produced; and the determination of the shelf life of the formulated products

6.6. Application methods

One of the main problems in releasing biocontrol agents is to find suitable methods of application that allow the agent to reach the target bio-constraint and to control it. Different approaches, such as the use of irrigation methods, application at the transplanting or seed coating will be developed using the different biocontrol agents, and their effect on efficacy and survival of agents can be considered.

Above or below ground, drip irrigation is often used for vegetable crops, with several advantages for the plants and the environment, such as saving of water, better management of nutrients, and fewer weed problems. The development of the plant root systems is influenced by water, and roots tend to grow close to the water application systems. Roots and unwanted microorganisms tend to clog or foul drip irrigation systems. Microorganisms might be an ideal way to control weeds and soil pathogens, as they would be conveyed directly in proximity of the roots. There might be great benefits from such methods of application, in terms of efficacy, reduced amounts of inoculum, protection from sources of inactivation (wind, dry air, UV light), no off-target spread, and homogeneity of control.

The best methods of application of the biocontrol agents will be determined and, in particular: the compatibility of irrigation systems with the application of living microbial agents will be evaluated. Optimized application technologies of wild type and modified *Fusarium* mycelial formulations will be developed in laboratory and greenhouse for the control of *Orobanche*. Other mycoherbicides for control of *Cirsium* sp. will be developed. The ability of phytotoxins to prevent irrigator clog by weed roots will be evaluated.

6.7. Assessment of field efficacy

The targets of the biocontrol agents are plant diseases and weeds that represent problems of utmost importance for many vegetable crops throughout Europe, in all climatic and environmental conditions, both in open fields and in greenhouses. The evaluation of the efficacy of the studied biocontrol agents is of strategic importance for determining their market size. In fact, the greater the possibility to use the organisms in different environmental conditions, crops and soil conditions, the wider will be the possibility to use the same formulation everywhere.

The ultimate selection of strains or combinations of strains, formulation, application technology and timing of the most promising microbial control organisms will be evaluated in field trials with cabbage, carrots, or lettuce.

6.8. Integration of biocontrol agents with other biocontrol agents and bioactive fungal metabolites

The combination of different pathogenic biocontrol agents, and of biocontrol agents with bio-active natural compounds is another strategy to improve their efficacy. Therefore, significant integrated research is likely to produce readily applicable protocols for effective exploitation of various biocontrol agents.

Several cell wall degrading enzymes and antibiotics play a major role in the complex biological processes involving *Trichoderma* strains for biocontrol (Harman & Kubicek, 1998). Some of these are applicable, both as proteins or genes, for the development of new defense strategies, transgenic and not, against phytopathogenic fungi (Lorito *et al*., 1998). It appears especially promising to use of mixtures of these enzymes (chitinases and glucanases) capable of degrading the fungal cell walls, since they are active on a wide spectrum of fungi. They are produced in large amounts by *Trichoderma* spp., are stable at room temperature, are capable of reaching efficacy levels similar to that of chemical fungicides.

Phytotoxic metabolites can weaken defence mechanisms of plants, rendering them more susceptible to pathogen attack. Thus, the application of toxins jointly with the pathogens could strongly enhance their bioherbicidal properties (Vurro *et al*., 2001b).

The use of combinations of biocontrol agents may also synergize the efficacy and reliability of biocontrol. For example, the combination of non-pathogenic *Fusarium* spp. plus a

Pseudomonad was more effective in controlling Fusarium wilts than either organism used singly (Alabouvette *et al*., 1996). Combinations of microorganisms must be fully compatible, i.e., the components of the microbial inoculant mixture must express their antagonistic activity against the target organism but not against each other. The metabolites produced by a component of the mixture must not interfere with growth and activity of the other components and possibly act synergistically with metabolites produced by these latter. Such an interaction has been shown with *Pseudomonas* lipopeptides and fungal cell wall-degrading enzymes of *Trichoderma* (Fogliano *et al*., 2002). A combination of antagonistic *Trichoderma* and non-pathogenic *Fusarium oxysporum* would be highly desirable because it would achieve better control of soil-borne pathogens of vegetable crops than either *Trichoderma* or *F. oxysporum* alone. However, *Fusarium oxysporum* can produce metabolites with antifungal activity (e.g. enniatins, fusaric acid) that could inhibit antagonistic *Trichoderma* spp. Conversely, some *Trichoderma* spp. produce isonitrile and peptide antibiotics (peptaibols), which inhibit fungal and bacterial growth.

6.9. Assessment of crop quality

Besides the ability of microbial agents to control bio-constraints, some strains may improve plant growth and productivity by other effects on the crops. Some *Trichoderma* strains have improved tolerance to stress, better induced resistance, and some solubilise and sequester of inorganic nutrients and enhanced uptake of nutrients by plants (Altomare *et al*., 1999; Bailey & Lumsden, 1998). The increased availability of both macro- and micro-nutrients to plants due to biocontrol agent activity may not only result in better plant growth, but also in a change in the general physiological state of the plant, which in turn influences its health, yield and most likely also the product quality in terms of nutritional factors, shelf life or taste. For this, comparative evaluations of nutritional value of biocontrol agents treated tomatoes vs. conventional products will be carried out.

The evaluation of the olfactory features of the crops has a strategic importance for the food companies that have to position their products correctly on the market and check consumer preferences or deal with special consumer sectors. Olfactory evaluations are also needed to check the effects caused by modifications in the production processes or the raw material, and to identify the ideal profile of a product by eliminating the defects. Objective measurements by panels (Scanlan, 1977) are valuable tools for development of high quality products.

7. Objectives relevant to the food quality and safety priorities of the EU

The first consequence of the improvement of the efficacy of biocontrol agents should be their wider use and consequently a reduction of the use of chemicals in Europe. Many European consumers have increased their interest in products obtained by organic or low input farming systems. Conversely, it is not cost-effective, and sometimes near impossible to produce healthy fruits and vegetables without means of controlling weeds, pathogens, and insect pests. Partially diseased or insect damaged fruits and vegetables often contain toxins produced by the plants to ward off the pests, or they contain the mycotoxins produced by pathogens of the crops, and these ‘natural’ chemicals often have human toxicities.

The use of alternative and environmentally friendly biocontrol systems for weed, disease and insect control in food production must be compatible with accepted concepts of food quality and safety. Management strategies to reduce chemicals are welcomed by both the public and by governments. For those reasons, the proposed project fulfils the primary objective of this EU Thematic Priority, that is to improve the health and well being of European citizens through a higher quality of food, with improved control of food production and of related environmental factors.

The quality and safety of food products is assured throughout a very long chain that begins in the field. The growth and the harvest of safe and high-quality crops is the first essential step in the production wholesome nourishment, and the use of technologies and strategies having the least possible inputs is of utmost importance to attain those quality products.

Despite the increased attention in biological control, the market of biocontrol microbes is presently still quite small. Some fungi are produced and sold by local companies, within a well defined niche, often without being registered as biocontrol agents, such as *Trichoderma* or bacteria species that are often registered and commercialized as bio-fertilizers or plant strengtheners. This is in part due to: the ease of registering microorganisms with vague 'growth promotion' activity compared to registration of a microbial pesticide; the lack of availability of efficacious agents; the lack of interest of the large companies in these products; the lack of knowledge of the potential of those microbes when used in different environments and crops; and, the lack of knowledge of the application methods. The availability of new and enhanced microorganisms and a well defined knowledge for their use can enlarge the market and can render those organisms more interesting as products for commercialization. One of the companies included in the project is a leader in the production and commercialization of biocontrol agents. It is actively involved in innovative aspects related to the technological properties of biological control agents that make a microorganisms suitable for use in the market. They have developed suitable media for growth and inoculum production, technologies to separate conidia, formulation, fitness, and microbial shelf life. This company can assist the group in the estimation of costs for production and formulation of microbial agents, together with the evaluation of registration procedures and the market size of those products. This will provide important support in developing technologies and opening markets for biocontrol agents.

The aim of the project fits well within the EU main objective of obtaining "safer and environmentally friendly production methods and technologies and healthier foodstuffs". In fact, the targets chosen (plant diseases and weeds) are among the worst bio-constraints of vegetable crops, acting at the soil level, and those are even more difficult to manage in low input and organic farming systems. The application of soil fumigants is one of the most efficient and widespread practices used to control soil bio-constraints' as discussed in the introduction. Most chemical control strategies are forbidden in organic agriculture, and the pesticides allowed in organic agriculture (sulphur, copper salts for fungi, pyrethrum, nicotine for insects, perchlorate, sulfuric acid, and soaps for weeds) are hardly benign to the environment, and are not cost-effective compared to the newest, more ecologically neutral synthetic pesticides. Interestingly, organic agriculture has not adopted the fermentation produced, natural herbicide, bialophos, which seems to meet all criteria for use as an organically produced material.

The European call required research to "harmonize methodologies for monitoring the effectiveness of the agents", and this request will be satisfied by the work-packages dealing with the "assessment of field efficacy" and with the "application methods", that will allow to identify the best conditions for the use of microbial agents, and to harmonize methodologies for their application.

The programme guidelines require noteworthy attention for “risk assessment”, and one work-package is specifically devoted to the evaluation of the possible undesired effects of the release of microbial agents, and systems to track the microorganisms in the soil after application or to mitigate the risk of their spread after the distribution will be developed. The influence of the introduction of antagonistic organisms on natural microbial communities will be considered, as well as the host specificity of weed pathogens and the potential to spread to crops or other non target plants.

Another requirement, that is a “large use of biotechnologies”, is fully met. These, developed for enhancement of efficacy, microbial production, fermentation, application, and risk assessment, could be further used in the future as guidelines to develop other biological control agents, attracting further interest of the scientific and industrial communities.

8. Potential impact

Concern over the evolution of fungicide-resistant strains of plant pathogens and of herbicide-resistant weeds, the loss of registration of some of the more effective pesticides or their phasing out, have generated an interest in the development of alternatives to synthetic agro-chemicals that are both effective and economically feasible. This has generated an increasing interest in biological control of plant diseases, pests, and weeds as an environmentally friendly practice to be used in conventional, low-input agriculture and organic farming.

The overcoming of some of the limits for the use of biocontrol agents can increase their use on horticultural, forest and field crops, in diverse habitats, helping in creating a “European” market.

The project will attempt to reinforce competitiveness of low-input agriculture and lower inputs. The end users to benefit from this project will be consumers asking for healthy food, organic farmers asking for alternative to agrochemicals, conventional farmers desiring to lower inputs, and European companies, especially small and medium enterprises developing or marketing biocontrol agents and their application technologies.

The broad use of molecular tools for precisely tracking the microbial strain released could be of great help in evaluating its real fate in the environment after the introduction, and mitigate the ephemeral worries of the public opinion about uncontrollable microbial dispersions.

Compared to previous project dealing with biological control funded by past EU frameworks, the project described differs because is not focused on just single targets, such us one noxious weed, or damping-off agents, it is not too narrow to protect a few crops, it is not only finalized to develop products such as commercial formulations or seed treatments, and covers important agriculture constraints interesting all European countries. It is hoped the results obtained could be easily adapted to different crops or exported and applied to different agents and against different targets.

9. Notes

The project ”Enhancement and Exploitation of Soil Biocontrol Agents for Bio-Constraint Management in Crops” (Acronym 2E-BCAs; contract FOOD-CT-2003-001687; http://www.2E-BCAs.org) is co-funded by the European Commission.

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