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Risk Analysis in the Release of Biological Control Agents

Antagonistic *Fusarium oxysporum* as a Case Study

Among biocontrol agents holding promise for practical application, saprophytic *Fusarium* spp., isolated from Fusarium-suppressive soils, have been extensively and successfully exploited for their activity against various formae speciales of *Fusarium oxysporum*, incitants of vascular wilts of numerous economically important crops. Saprophytic *Fusarium* spp. could represent a very useful tool in Fusarium wilt management, considering that on most crops control of the disease is difficult, often incomplete, and still relies on the integration of different control measures. The high level of disease control provided, coupled with the consistency of the results obtained, make saprophytic *Fusarium* spp. good biocontrol candidates on crops such as tomato, melon (1), carnation (18,19), cyclamen (34), and basil (33). In Italy, research has focused on the biocontrol activity of antagonistic *F. oxysporum* against *F. oxysporum* f. sp. *dianthi* on carnation. Antagonistic *Fusarium* spp. isolated from the rhizosphere of carnation plants grown in suppressive soils in Italy are rhizosphere competent with a high ability to colonize roots (20). Competition with the pathogen for occupation of infection sites is one of the suggested mechanisms of action of these antagonists (9). Strain improvement has been attempted by mutagenesis (19), protoplast fusion (32), and transformation (28). These efforts yielded several antagonistic strains, naturally occurring or genetically transformed, ready for commercial exploitation. A commercial formulation based on *F. oxysporum* strain 251/2 is now undergoing the registration process (2).

However, large-scale introduction of biocontrol agents, genetically manipulated or not, requires careful evaluation of their environmental impact (5,11,47). Biocontrol agents must be genetically stable after introduction in the soil and should be able to survive and maintain themselves for adequate biocontrol activity, but must not interfere with the resident microflora. The potential hazards posed by an artificially increased population have been evaluated for bacteria (21), entomopathogenic fungi (25), and mycoherbicides (12,44,53). Much less information is available in the case of fungal biocontrol agents of phytopathogens.

Before the large-scale introduction of antagonistic *F. oxysporum,* a careful evaluation of the potential of genetic transfer between strains is necessary, because of the close genetic relatedness of the antagonists to pathogenic *F. oxysporum*.

This paper gives an overview of the risk analysis study carried out for use of antagonistic *F. oxysporum* to control Fusarium wilts. The analysis consisted of laboratory, closed microcosm, and glasshouse experiments, and took into consideration biological, ecological, and genetic aspects.

Strains Used, Induction of Markers, and Fingerprinting

A requisite to any study related to the behavior of artificially introduced microorganisms in the environment is the ability to track them. Biochemical markers and DNA fingerprinting were developed which allowed us to follow individual biocontrol strains of *Fusarium* after their release in the soil. The study included naturally occurring saprophytic *F. oxysporum*, UV-induced mutants, hybrids derived from protoplast fusions, and transformed strains.

Naturally occurring, wild-type (wt) antagonistic *F. oxysporum* (strains 141 wt, 233 wt, 245 wt, 251 wt, and 257 wt) were isolated from the rhizosphere of carnations grown in *Fusarium*-suppressive soil. Benomyl-resistant mutants (233/1, 251/1, 251/2, 251/3, and 257/1) were obtained by a 30-min UV treatment from 233 wt, 251 wt, and 257 wt, respectively. The induction of benomyl resistance by mutagenesis did not reduce the biocontrol potential of the isolates, making this technique feasible for developing strains to be used in combination with benzimidazole fungicides under integrated control strategies (19). An orange-pigmented mutant (141 C7.2) and a dark red-pigmented mutant (233/1 C5) were derived from UV-treated strains 141 wt and 233/1, respectively (Fig. 1).

Hybrid strain FI-11 is derived from an intra-strain protoplast fusion of auxotrophic mutants 251/3 *ile*$^-$ and 251/3 *lys*$^-$, which were selected after a 2-h exposure of conidia from mutant 251/3 to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (500 μg ml^{-1}). Several benomyl- and hygromycin B-resistant transformants were obtained from different antagonistic strains (251/2, 233/1 C5, 141 wt, and 141 C7.2) of *F. oxysporum* by PEG-mediated insertion of the *benA* gene from *Aspergillus nidulans* and the *hph* gene of *Escherichia coli*, respectively (Fig. 2).

Molecular fingerprinting of antagonistic *Fusarium* spp. was attempted by means of electrophoretic karyotyping and by random amplification of polymorphic DNA (RAPD). For the electrophoretic karyotype determination, intact chromosome-size DNA of antagonistic *Fusarium* spp. was isolated from fungal protoplasts, embedded in agarose, separated by contour-clamped homogeneous electric field (CHEF) gel electrophoresis (7,52), and visualized under UV light after staining with ethidium bromide (29). In order to generate RAPD profiles, several 10-mer and 12-mer oligonucleotides were tested as primer sequences, and DNA amplification was performed by following standard methods (54,55). Polymorphism was observed both in the electrophoretic karyotype and in the RAPD profile of different antagonistic strains or pathogenic isolates of *Fusarium* spp. (Figs. 3 and 4). The karyotype and the amplified DNA profiles were highly reproducible when DNA was extracted from independently isolated colonies of the same strain, thus making these techniques suitable for tracking individual strains once released in the environment (29,30). In our studies, the use of

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fungicide resistance and color markers, in combination with electrophoretic karyotyping or RAPD markers, allowed the unequivocal recognition of selected antagonistic strains several months after their introduction in both disinfested and nondisinfested soils and in the plant rhizosphere (see below). Other suitable tracking techniques were more recently developed for antagonistic *F. oxysporum*. These include the use of polyclonal antibodies and β-D-glucuronidase (GUS)-marked strains (17), restriction fragment length polymorphism (RFLP) analysis of total DNA after hybridization with a random probe, enterobacterial repetitive intergenic consensus (ERIC-) or repetitive extragenicpalindromic elements (REP-) polymerase chain reaction (PCR) based fingerprinting, and restriction fragment analysis of PCR-amplified ribosomal intergenic spacers (16).

Risk Factors Considered

Persistence and survival. The ability of saprophytic *F. oxysporum* to survive and potentially flourish beyond the time period and place originally intended was evaluated both in closed microcosms and under greenhouse conditions. Experiments with antagonistic *Fusarium* spp. were performed in soil microcosms (1-liter capacity) filled with either natural or steam-disinfested soil infested with a conidial suspension of each antagonist (5×10^5 CFU g^{-1} of soil). Microcosms were kept in sealed polythene bags and maintained in a glasshouse at 20°C. Soil samples were collected weekly after 1 to 20 weeks from release, and serial dilutions were prepared and plated onto Komada *Fusarium*-selective medium (23). The population of *Fusarium* spp. strains generally decreased sharply once they were introduced into the soil environment, reaching a stable concentration of 10^3 to 10^4 CFU g^{-1} of soil after 6 to 10 weeks (Fig. 5). Because of the presence of resident competitors, survival in natural soils was less than that observed in disinfested soils. No significant differences were observed in the population dynamics of transformant, hybrid, mutant, or wild-type strains under any of the conditions tested (27). The number of CFU of benomyl- or hygromycin B-resistant transformants isolated on selective medium amended with 10 μg ml^{-1} of benomyl or with 100 μg ml^{-1} of hygromycin B was similar to the number of CFU isolated in the absence of fungicides, showing that both phenotypes are highly stable, suitable markers to track genetically modified *Fusarium* spp. in soil. A mixture of genetically altered antagonistic strains and wild-type strains did not influence the population dynamics and the survival ability of any of the strains. Similarly, coinfestation with a pathogenic strain of *F. oxysporum* f. sp. *dianthi* and the presence of its host plant, carnation, did not significantly modify the behavior of the antagonistic transformant in soil (Fig. 6; 27).

One physical factor to be taken into consideration in any large-scale release of microorganisms is water-induced dispersal. Transport under the influence of water depends not only on the extent and rate of water input, but also on factors inherent in the microorganism (e.g., cell size, type of inoculum, spore concentration) and in the soil (e.g., texture, pH, clay mineral content) (36). Moreover, temperature influences water transport (50), as do cultural practices and resident microflora.

Dispersal by water of benomyl-resistant biocontrol strain 251/2 of *F. oxysporum* from two different soils was evaluated in closed microcosms under laboratory conditions. Rigid polyvinyl chloride tubes (25 cm long, 19.5 cm internal diameter) were used. A funnel with the same external diameter as the tubes, filled with 500 or 1,000 g of sieved soil (depending on soil bulk density), was tightly fixed beneath each column, with a discharge tube leading into a sterile 2-liter plastic beaker. A fine cotton net plugged the bottom of the column to prevent soil from escaping. Soil was infested with one talc preparation and one commercial alginate formulation of the antagonist at 10^5 CFU g^{-1} of dry soil. Soil columns were incubated at 25°C through the experiments. In sandy loam soil, recovery of antagonistic *F. oxysporum* from dispersal water, either as chlamydospores in talc powder or as formulated alginate pellets, was observed for periods longer than 60 days (Fig. 7). In a soil-based potting mixture (2 parts soil and 1 part peat moss, vol/vol), release in water was observed only when infestation was carried out with alginate pellets. Thirteen to 15 days after soil infestation, it was not possible to recover propagules from water dispersed through soil infested with talc powder (Fig. 8). As already observed in the case of bacteria (4,50), probably no adsorption of antagonistic *Fusarium* spp. onto sandy soil takes place, while fungal cells are adsorbed onto clay minerals. *F. oxysporum* survival and persistence followed a similar pattern in both soils (M.

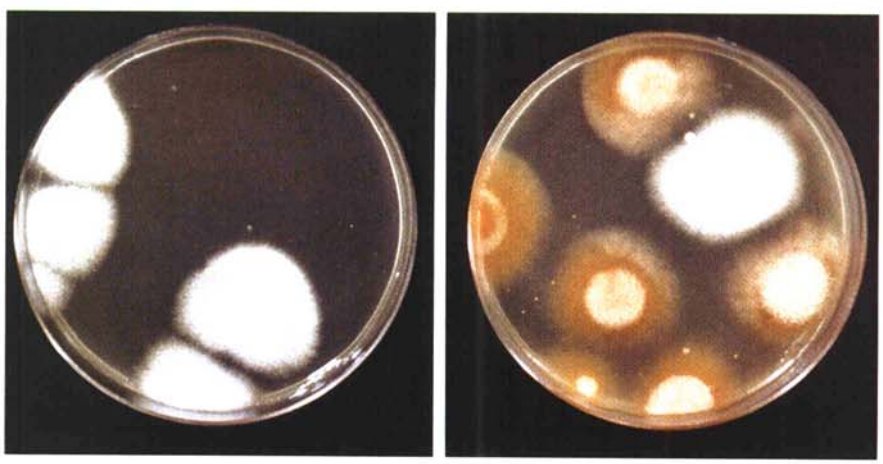

Fig. 1. Orange- and dark red-pigmented mutants of antagonistic *Fusarium oxysporum* are easily recognized in mixed culture with the wild-type strain (white).

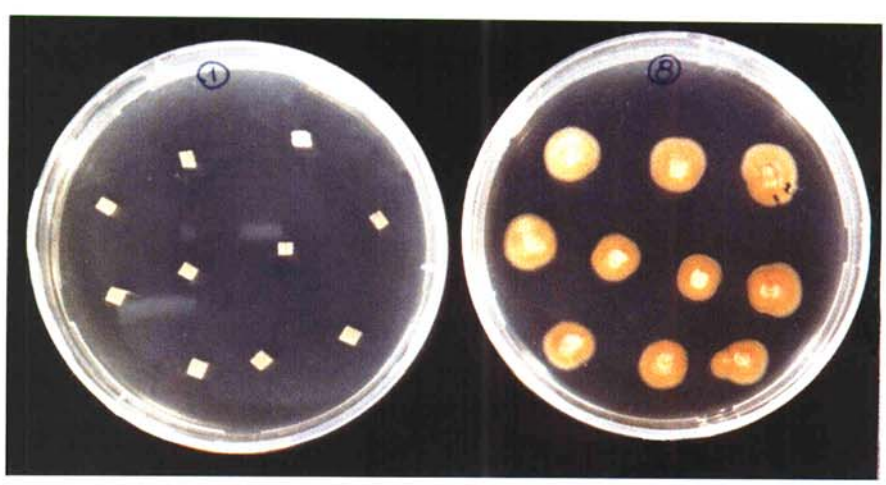

Fig. 2. Benomyl-resistant transformants obtained by polyethylene glycol (PEG) mediated insertion of the *benA* gene of *Aspergillus nidulans* into an orange-pigmented mutant of antagonistic *Fusarium oxysporum*. Agar medium contains 20 mg ml^{-1} of benomyl.

Mezzalama et al., *unpublished*) (Figs. 7 and 8). Encapsulation of *Fusarium* spp. in alginate beads enhances their survival capability (27).

Effects of introduced microorganisms on microbial communities. Newly introduced antagonistic microorganisms may cause both qualitative and quantitative alterations in the microbial community structure. This is considered to be one of the most difficult aspects to evaluate in risk assessment because of the number of studies required to estimate probable effects resulting from controlled or uncontrolled release (5,21). Displacement of native microbial groups can be dramatic if introduced organisms possess a high fitness, and unwanted effects may occur when the displaced microflora plays an important role in the geochemical cycling of nutrients, thus having broad consequences on the ecosystem. To estimate the effects of an introduced antagonist on microbial communities, the population dynamics of specific functional groups should be evaluated, e.g., those linked to the processing of nitrogen, sulfur, and phosphorus, nitrogen-fixing organisms, and mycorrhizae. To evaluate the quantitative influence of antagonistic *Fusarium* spp. on resident microbial communities, wild-type and genetically manipulated antagonistic strains were introduced into natural soil microcosms, and the total content of fungi, actinomycetes, and bacteria was assessed weekly. Plastic tubes (12 cm long, 2.8 cm internal diameter) filled with sieved natural soil were used as closed microcosms. The

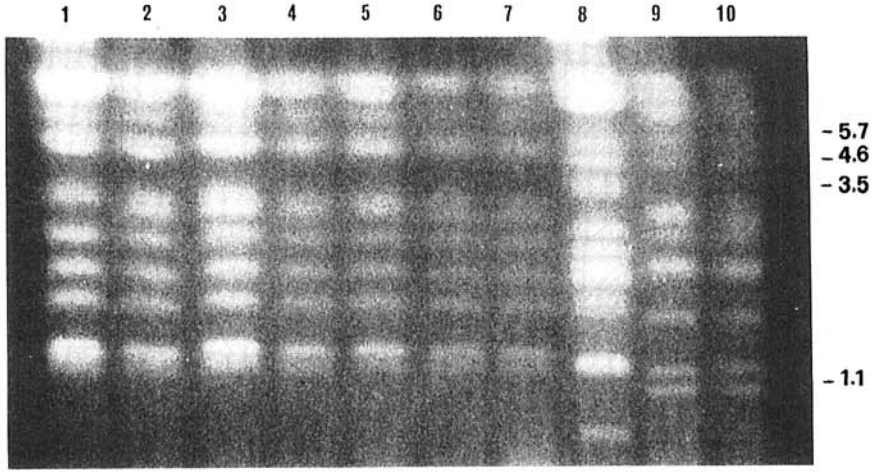

Fig. 3. Electrophoretic karyotype variability in antagonistic *Fusarium oxysporum*. Individual strains may be tracked after release in the environment. From left to right: (1) 251 wt (wild type); (2 to 7) 251/3 from 0 to 5 months after introduction into soil; (8) 141 wt; (9) 233 wt; (10) 233/1. DNA size standards (Mb) are given on the right.

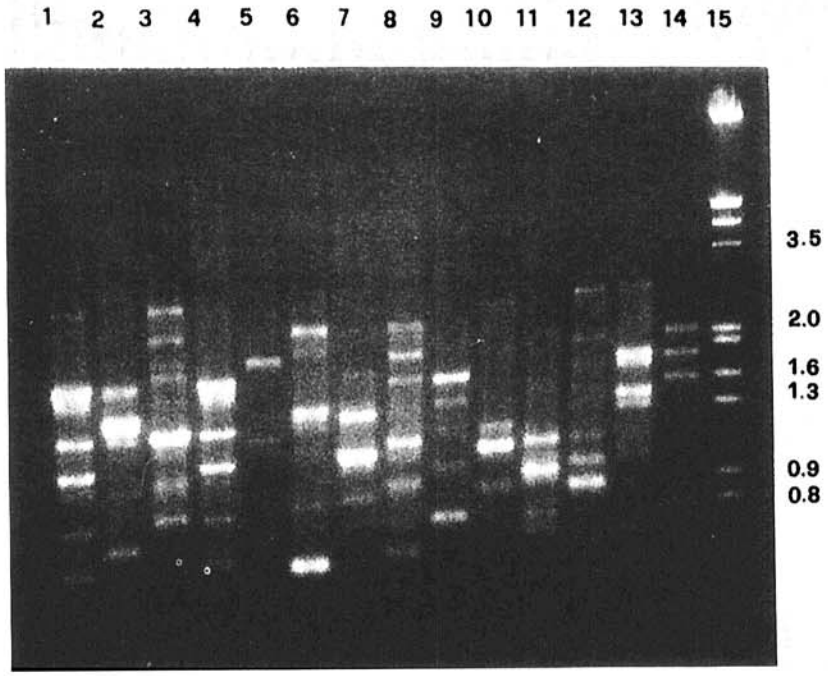

Fig. 4. RAPD profiles of *Fusarium* spp. can be effectively used for strain fingerprinting. Polymorphic bands of amplified DNA from (1) *F. oxysporum* f. sp. *cepae*; (2) *F. o.* f. sp. *dianthi*; (3) *F. o.* f. sp. *basilici*; (4) *F. o.* f. sp. *radicis-lycopersici*; (5) *F. o.* f. sp. *lycopersici*; (6) *F. o.* f. sp. *culmorum*; (7) *F. sporotrichioides*; (8) *F. tabacinum*; (9) *F. moniliforme* (TF4); (10) *F. moniliforme* (912); (11) *F. roseum*; (12) antagonistic strain of *F. oxysporum* 233 wt; (13) antagonistic strain of *F. oxysporum* 141 C7; (14) DNA from *Dianthus* sp.; (15) bacteriophage lambda DNA digested with *Eco*RI and *Hin*dIII. Primer sequence: 5'-ATTGCGTCCGAG-3' (30). DNA size standards (Kb) are given on the right.

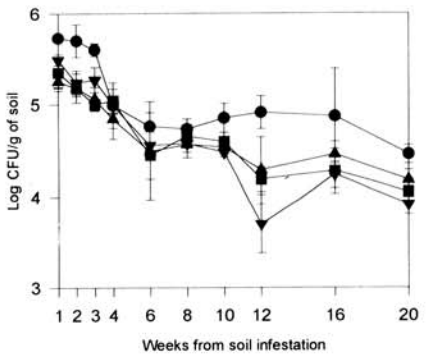

Fig. 5. Population dynamics of an orange-pigmented antagonistic strain of *Fusarium oxysporum* (141C7.2 ■) and of a benomyl-resistant strain genetically manipulated (T8/141RB ●) released alone or in mixture (mix141C7.2 ▼; mixT8/141RB ▲) in closed microcosms (1-liter capacity) containing disinfested soil at 10^5 CFU g^{-1} of dry soil. Isolations were carried out on Komada *Fusarium*-selective medium (23). Each data point represents the mean of 5 replications of 10 plates each; vertical bars indicate standard deviations.

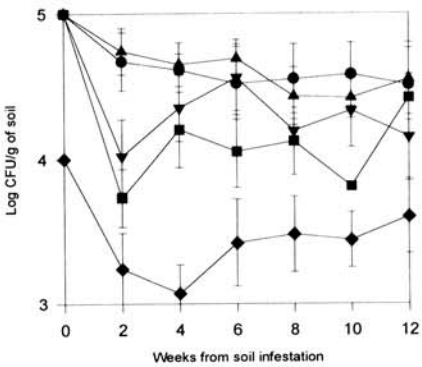

Fig. 6. Population dynamics of an orange-pigmented antagonistic strain of *Fusarium oxysporum* (141C7.2 ■) and of a benomyl-resistant strain genetically manipulated (T8/141RB ●) released at 10^5 CFU g^{-1} of dry soil, alone or in mixture (mix141C7.2 ▼; mixT8/141RB ▲), in closed microcosms (1-liter capacity) in the presence of pathogenic *F. o.* f. sp. *dianthi* (◆) and of the host plant (carnation cv. Indio). Isolations were carried out on Komada *Fusarium*-selective medium (23). Each data point represents mean of 5 replications of 10 plates each; vertical bars represent standard deviations.

plate count method was used to assess the population dynamics of fungi, actinomycetes, bacteria, and fluorescent pseudomonads on selective and semiselective media. The quantitative evaluation made with this method showed that the introduction of wild-type and genetically manipulated antagonistic strains of *F. oxysporum*, released alone or in mixture, does not interfere with the microbial equilibrium of a natural soil in closed microcosms. Moreover, no interaction was observed with the population of fluorescent *Pseudomonas* spp., well-known for their positive effect in the soil (Fig. 9). The same results were obtained when antagonistic *F. oxysporum* was released in the rhizosphere of cucumber plants. Antagonistic *F. oxysporum* actively colonized the rhizosphere of cucumber grown in nondisinfested soil, but the total number of propagules of indigenous fungi and bacteria recovered in the rhizosphere was not affected by the presence of the antagonists.

Similarly, although the introduction of genetically modified *P. aureofaciens* in the rhizosphere (and the phylloplane) of wheat did not interfere with the indigenous microbial population of fungi, actinomycetes, and yeasts, it had some effect on the population of fluorescent pseudomonads. However, since this effect was evident for only a short length of time, the release of such microorganisms was not considered to be a substantial risk (13). On the contrary, the release of wild-type and genetically altered *Erwinia carotovora* did interfere quantitatively with the indigenous microbial community (35).

Dipping roots in a conidial suspension of manipulated and nonmanipulated strains of antagonistic *F. oxysporum* did not influence root colonization (percent mycorrhized roots or number of nodules cm^{-1} of root) and plant dry weights of *Ocimum basilicum* and *Pisum sativum* by either *Glomus versiforme* or *Rhizobium leguminosarum* and dry weights of treated and nontreated plants (data not shown).

Also in the case of *Gliocladium virens* and *Pseudomonas* spp., no negative effect on the root colonization of cucumber and onion by the vesicular arbuscular mycorrhizal fungi *Glomus intraradices* and *G. etunicatum* was observed (37,38).

The effects on total soil biomass, accepted as a measurable marker for a perturbed ecosystem (42), were investigated by evaluating CO_2 production, hydrolysis of fluorescein diacetate, ATP content, and biomass carbon and phosphorous. Several authors (40,43) stress the need of adopting more than one technique for measuring soil biomass, since each method has some limitations (40). Among the methods we adopted, the chemical evaluation of evolving CO_2 in three different types of soil infested with antagonistic strains of *F. oxysporum* showed a significant increase within 10 to 13 days after soil infestation, followed by a decrease to control values, indicating that gross metabolic activity was not altered (M. Mezzalama et al., *unpublished*). Our results confirm that it is wise to use more than one independent method, wherever possible, in order to overcome natural variability and assess whether the release of an antagonist does actually modify the soil ecosystem.

Stability and genetic transfer. The mitotic stability of genetically manipulated antagonists can be determined through

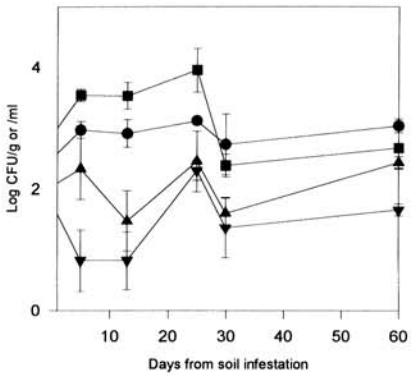

Fig. 7. Survival in soil and dispersal in water of antagonistic benomyl-resistant *Fusarium oxysporum*, strain 251/2RB, formulated in talc powder (■ in soil; ▼ in water) in alginate pellets (● in soil; ▲ in water) in a sandy loam soil (pH 8.02) infested with 10^5 CFU g^{-1} of soil and incubated at 25°C for 60 days. Isolations were carried out on Komada *Fusarium*-selective medium (23) amended with 10 μg ml^{-1} of benomyl. Each data point represents mean of 4 replications of 10 plates each; vertical bars represent standard deviations.

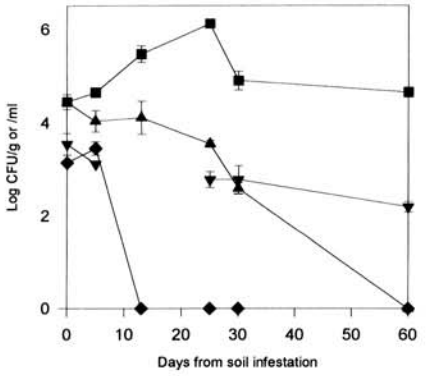

Fig. 8. Survival in soil and dispersal in water of antagonistic benomyl-resistant *Fusarium oxysporum*, strain 251/2 RB, formulated in talc powder (■ in soil; ▼ in water) or in alginate pellets (● in soil; ▲ in water) in a soil-based potting mixture (2 parts soil, 1 part peat moss vol/vol, pH 7.2) infested with 10^5 CFU g^{-1} of soil and incubated at 25°C for 60 days. Isolations were carried out on Komada *Fusarium*-selective medium (23) amended with 10 μg ml^{-1} of benomyl. Each data point represents mean of 4 replications of 10 plates each; vertical bars represent standard deviations.

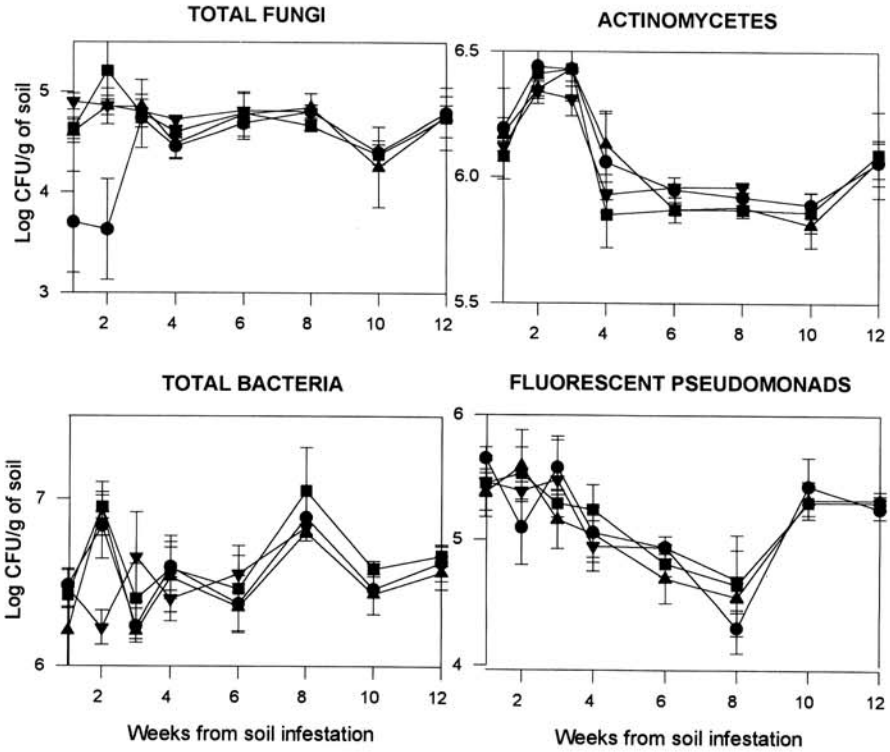

Fig. 9. Population dynamics of fungi, actinomycetes, bacteria, and fluorescent pseudomonads in a natural soil infested or not (●) with antagonistic orange-pigmented strain of *Fusarium oxysporum* (141C7.2 ▲) and a benomyl-resistant strain genetically manipulated (T8/141RB ■) released alone or in a mixture (▼) at 10^5 CFU g^{-1} of dry soil in closed soil microcosms (300-ml capacity). Each data point represents mean of 3 replications of 10 plates each; vertical bars represent standard deviations.

comparative Southern analysis of DNA extracted from cultures derived from spores obtained before and after their release to the environment.

Both hygromycin B resistance and mitotic stability of 10 hygromycin B-resistant transformants of antagonistic *F. oxysporum* derived from mutant 233/1 C5 were evaluated in vitro in the absence of antibiotics and after release in soil microcosms. Hybridization with the complete transforming plasmid suggested that integration to chromosomal-sized DNA had occurred in a multiple-tandem array at multiple sites (Fig. 10). After four rounds of vegetative growth without selective antibiotic pressure or 4 weeks after release in soil microcosms, some of the transformants were mitotically stable, while others showed various changes in the integration pattern (Fig. 10) and one transformant had lost the ability to grow in the presence of hygromycin B (Fig. 10; Migheli et al., *unpublished*).

The results obtained with some antagonistic *Fusarium* spp. are in agreement with those obtained with transformed strains of antagonistic *Trichoderma harzianum*, which were shown to be mitotically stable after release into the soil (39) or the phylloplane (31).

The possibility of genetic transfer from antagonistic to pathogenic strains of *Fusarium* spp. and vice versa was identified as one of the main risks in the release of such biocontrol agents in the environment. Before developing and implementing such a biocontrol method, it is necessary to assess this hazard posed by artificially increased populations of saprophytic *F. oxysporum* in the soil. The risk should be more acceptable for *F. oxysporum* strains, which lack a sexual reproductive system, as the frequency of genetic exchanges is reduced in comparison to sexually reproducing species. Moreover, the fact that transforming DNA sequences are usually integrated into chromosomes in filamentous fungi (48) significantly reduces their mobility and the possibility of transfer. However, the occurrence of genetic drift following parasexual mechanisms between resident and introduced *F. oxysporum* strains or via their interaction with soil microflora or with the host plants cannot be excluded a priori and deserves further study.

The acquisition of benomyl resistance by sensitive antagonistic strains of *F. oxysporum* from resistant strains can be routinely accomplished by protoplast fusion under laboratory conditions at a frequency of 10^{-4} to 10^{-6} (32). In order to evaluate the frequency of horizontal transfer of benomyl resistance between newly introduced strains and other *Fusarium* spp. strains, a benomyl-resistant transformant was co-cultured in vitro with a benomyl-sensitive *F. oxysporum* mutant marked by orange pigmentation. Conidial suspensions and mycelium were used in the experiments. The development of colonies on PDA amended or not amended with benomyl was recorded after prolonged liquid culture in the absence of selective pressure. The same strains were introduced as conidial suspensions in soil microcosms in the presence or absence of carnation plants in order to evaluate the possibility of genetic transfer of benomyl resistance under natural conditions. Orange colonies were never isolated on benomyl-amended substrate from soil in a 6-month experiment (26). Additional auxotrophic markers were used in order to assess the frequency of nutritional complementation between *F. oxysporum* strains, but no evidence for genetic horizontal transfer was obtained under either laboratory or natural conditions. Different families of transposable elements have been identified in phytopathogenic *F. oxysporum* (10), and their potential role as sources of genetic instability and horizontal transfer in phytopathogenic, saprophytic, and antagonistic *F. oxysporum* is now under investigation.

Pathogenicity and toxicology. Antagonistic strains must be harmless not only toward the plant on which they are applied, but also on nontarget and cultivated plants, which could be exposed intentionally or unintentionally to their propagules. Pathogenicity studies are critical for both genetically modified and wild-type biocontrol strains of *Fusarium* spp. because of phylogenetic similarities with the phytopathogenic formae speciales. Several effective wild-type, mutant, hybrid, and transformed strains of antagonistic *Fusarium* spp. were tested for pathogenicity in a contained environment over a large array of cultivated and noncultivated plants grown under protected conditions (Table 1). After application of the antagonists as either a conidial or chlamydospore suspension in soil (10^5 CFU ml^{-1} of soil) and as a root dip (10^6 CFU ml^{-1}) before transplant, no typical disease symptoms (e.g., growth reduction, yellowing, wilting, vascular discoloration) or any other negative effects were observed on any of the tested plant species.

A number of standard toxicological tests, developed for evaluating the presence of toxic or mutagenic metabolites in homogenized *F. oxysporum* cells and in the culture filtrates (3), have been conducted. These tests include inhibition of root development in tomato germlings, radial growth of the fungus *Geotrichum candidum*, toxicity towards larvae of the crustacean *Artemia salina*, and genotoxicity on germ cells and epithelial liver cells of Chinese hamster. The results obtained thus far indicate that, among 10 strains tested, only one wild-type strain and one benomyl-resistant transformant induced significant increases in sister chromatid gaps on a Chinese hamster ovary cell line. However, such an increase was observed only after the metabolic conversion of culture broth by rat S9 occurred, while no direct genotoxic effects of the culture filtrates were detected (6). Allergenicity as a consequence of routine manipulation of these strains in the laboratory was never observed.

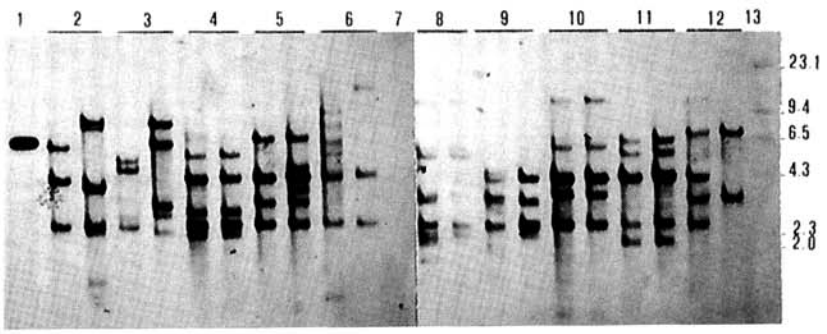

Fig. 10. Southern blot hybridization analysis of 10 hygromycin B-resistant transformants (2 to 6 and 8 to 12) deriving from the antagonistic *Fusarium oxysporum* mutant 233/1 C5 (lane 7). DNAs from the transformed strains grown in vitro in the presence of hygromycin B in the medium (left lane of each pair) or after four rounds of growth in the absence of selective pressure (right lane of each pair) were digested with the restriction enzyme *Eco*RI and hybridized against the label linearized plasmid vector PAN7-1 (lane 1), used for transformation. Lane 13 corresponds to bacteriophage lambda DNA digested with *Hin*dIII. DNA size standards (Kb) are given on the right.

Table 1. List of some crop plants and cultivars inoculated with *Fusarium* spp. for pathogenicity testing

Plant	Cultivar(s)
Basil	Genovese, Fine Verde
Bean	Borlotto, Sciabola Rossa, Nano
Carnation	More than 100 cultivars
Cucumber	Ibis, Marketer
Cyclamen	Rosamunda, Scarlet, Finlandia
Melon	Supermarket, Cantalupo
Parsley	Gigante d'Italia
Pea	Meraviglia
Tomato	Marmande, Bonny Best
Zucchini	Maya

Containment Methods

Methods to monitor an immediate decline in the population of artificially introduced biocontrol agents are needed in the event of unwanted, unplanned escapes. Before deliberate release of genetically manipulated or wild-type biocontrol strains of *Fusarium* was initiated, several kinds of containment systems were adopted. Physical barriers (glasshouses, growth chambers, enclosures, sealed windows, air locks, filters) prevented the escape and dispersal of the organism. Contaminated waste (solid and liquid growth media, petri dishes, infested soil), tools, and garments were decontaminated before dispersal or re-use, and the tested organisms were autoclaved at the end of the experiments. Effective chemical or physical control measures are available for soilborne fungi. Conventional control measures for soilborne plant pathogens, such as soil–substrate steaming or fumigation with methyl bromide or other fumigants, can successfully and rapidly abate the population of newly introduced *Fusarium* spp. under commercial glasshouse conditions (Gullino et al., *unpublished*). Use of benzimidazoles, on the contrary, can only provide a partial reduction in the population of benzimidazole-sensitive antagonistic *Fusarium*, while they are ineffective against the benzimidazole-resistant mutants of the antagonist. Even in the case of sensitive *Fusarium* spp. strains, benzimidazoles cannot provide the quick-kill effect needed in case of escape. Among other available fungicides, only prochloraz (Octave, 50% a.i., AgrEvo, Germany) was effective in reducing the population of antagonistic *Fusarium*.

In the case of engineered *Pseudomonas syringae*, *P. fluorescens*, and *Erwinia herbicola* applied to bean leaves grown in field plots, burning plus tillage proved the most effective decontamination treatment, producing the most significant reductions in bacterial populations (14).

Outlook

The increased interest in biological control of plant diseases has led to the testing of many naturally occurring microorganisms for experimental purposes. Release of wild-type strains apparently has not resulted in significant perturbations of the habitats into which they have been introduced, but the majority of these studies have not sought to address this possibility (56).

Our studies were undertaken with the aim of evaluating environmental risks related to the large-scale introduction of antagonistic *F. oxysporum*, which are considered by many as feasible biocontrol candidates against Fusarium wilts of several economically important crops. The work was performed with both wild types, naturally occurring saprophytic strains of *F. oxysporum*, and genetic recombinant strains. Genetic stability, potential genetic transfer from one strain to another, pathogenicity to host and nonhost plants, and disruption of the ecosystem were identified as potentially the most critical hazards.

Suitable marker systems and tracking methods were developed, which proved reliable and sensitive for monitoring the establishment, movement, and fate of antagonistic *Fusarium*. Indeed, the availability of such methods is helpful, not only for risk assessment purposes, but also for a better comprehension of the mechanism(s) of action of these antagonists, as in the case of GUS-transformants, which were used to study the competition between pathogenic and nonpathogenic strains of *F. oxysporum* at the rhizosphere level (17).

Antagonistic *Fusarium* are genetically stable and able to survive long enough to provide satisfactory control of Fusarium wilt on several crops for 4 to 8 months. Interestingly, in disinfested and natural soil, and in the rhizosphere of plants, a decline in the population of introduced antagonistic *Fusarium* was observed, thus indicating minimal risk of buildup in the environment.

Our studies with antagonistic *F. oxysporum* confirmed what we observed in the case of bacteria, where the population of nonpathogenic, nonadapted bacterial isolates usually decreases once they are introduced into a new environment, reaching undetectable levels within a few months after their release (15,51). The greatest vertical movement of antagonistic *Fusarium* spp. throughout water transportation was found in lighter, sandy soils.

The lack of any detectable negative effect on the resident microflora is a positive feature for the practical application of antagonistic *Fusarium* spp. The great buffering capacity and resistance to change of soils, which has been generally attributed to the abundance and diversity of life, should serve to prevent major shifts in microbial communities, even after introduction of large amounts of such biocontrol agents.

The behavior, persistence, and dispersal of wild-type antagonistic *F. oxysporum* strains was similar to that of UV-induced, benzimidazole-resistant mutants, hybrids, and genetically transformed strains. This is in agreement with studies conducted on genetically altered bacteria (56).

Closed microcosms proved a reliable method for collecting primary data on the behavior of antagonistic *Fusarium* spp. and their impact on the ecosystem under relatively realistic, albeit restricted, conditions. However, mesocosm and field studies are necessary in order to confirm conclusions from microcosms studies (45,46). The results concerning survival of wild-type and genetically altered antagonistic *Fusarium* spp. obtained from microcosm studies were confirmed under larger scale, greenhouse experiments (27). Furthermore, in the case of transgenic bacteria, spread and survival in the field could be predicted by microcosm studies (8,22,24).

The benzimidazole resistance gene selected to evaluate the possibility of genetic exchange between strains is a convenient selection marker and is well-studied at the molecular level. Any possible transfer of fungicide resistance genes to other microorganisms and particularly to plant pathogens was identified as an unacceptable risk. Among available fungicides, benzimidazoles provide the best control of Fusarium wilts; if benzimidazole resistance is transferred to pathogenic *F. oxysporum*, serious practical consequences can be expected. Genetic exchange did not occur even under the most conducive experimental conditions. Although the possibility of genetic exchange cannot be completely excluded, our results provide sufficient evidence for considering the introduction of antagonistic *F. oxysporum* into agroecosystems to be safe with minimal risks. Moreover, conventional soilborne disease control methods such as steaming and fumigation could be used to provide a rapid and effective decontamination procedure in the event of unwanted escape of saprophytic *F. oxysporum*.

Results obtained in numerous studies over the years lead us to conclude that the large-scale introduction of saprophytic *F. oxysporum* as biocontrol agents against Fusarium wilts will not represent an environmental hazard to agricultural and natural ecosystems. Such antagonists belong to a well-characterized species and have been tested extensively for years with no evidence of problems for agricultural crops and wild plants. It seems reasonable that biocontrol agents should be subject to scrutiny before they are released on a large scale; however, when competition is involved, as in the case of saprophytic *Fusarium*, and as often happens with naturally occurring microorganisms, less stringency should be required (25). Additionally, some risk factors can be considered of minor importance or even disregarded in the case of well-characterized species (41).

It seems evident that the effects of introduced genetically transformed strains on the ecosystem should be compared with the effects of the parent, wild-type strains. Only effects significantly different from those of parent strains should be taken into account when the biosafety of released genetically transformed strains is to be evaluated (49).

We hope that our studies provide evidence that risk assessment studies of biocontrol agents applied against plant diseases can be done scientifically by taking into account biological, ecological, and genetic aspects. On the basis of such studies, restrictions, if necessary, can be imposed on documented, not conjectural risk.

Interestingly, risk analysis studies also can generate information relevant to practical application of biocontrol agents.

The renewed emphasis on many aspects of microbial ecology, an often-neglected area, represents a positive output of microbial risk-assessment studies. Beyond the theoretical aspects, there are many practical implications and benefits. A better understanding of the ecology of antagonistic fungi and of their interactions with other microorganisms will permit more responsible use, thus fostering the implementation of biocontrol. Moreover, such studies can take advantage of other disciplines in bringing new methods for studying complex systems and especially for determining the impact of one species on another.

We hope to have shown that antagonistic, saprophytic strains of *F. oxysporum* can be released without a deleterious perturbation of a given habitat, and that their release, in combination with a thorough study of their ecology, can contribute to the implementation of successful biocontrol of Fusarium wilts on economically important crops.

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