

## Problems Posed by the large Scale Application of Microorganisms for Biological Control of Soil-Borne Plant Pathogens\*

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### Abstract

Many microorganisms have been described for their antagonistic activities against plant pathogenic fungi. Extensive studies have been conducted to prove their efficacy in microplots or under greenhouse conditions, but only a few of them have been tested in full-scale field experiments.

In fact, to experiment on a large scale, several technological problems have to be solved. Having selected an efficient strain, large amounts of inoculum have to be produced. New technical procedures have to be developed, especially for filamentous fungi. Then, it is necessary to dry this inoculum; to formulate it so that it can be easily stored, transported and subsequently applied to seeds or soil; and test it under different climatic conditions, in different soils naturally infested with the pathogenic fungus, over a period of several years. Before it can be used on a commercial scale it is also necessary to prove that this antagonistic microorganism is harmless to men and environment.

All these steps require financial and technical investments not usually found in a single laboratory; therefore cooperative work with other laboratories, industry, or farmers is necessary.

These problems and proposed solutions will be illustrated using the following examples of biological control of:

- take-all with hypoaggressive strains of *Gaeumannomyces graminis* var. *tritici*
- sclerotinia root rot with strains of *Trichoderma*
- fusarium wilts with strains of nonpathogenic *Fusarium*.

Keywords: biological control, antagonistic microorganisms, soil-borne plant pathogens

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## 1. Introduction

A review of the literature shows that numerous microorganisms have been proposed as agents to control plant diseases. Some of them have been extensively studied in the laboratory to understand the mechanisms by which they control diseases. They express relationships of antibiosis, competition or hyperparasitism harmful for the pathogenic fungi, and some induce or stimulate the defense of the plant (phenomenon of cross-protection). Although their efficacy has been proven in microplots or greenhouse experiments, they usually have not been used on a commercial scale. One must examine the reasons for this failure; in fact, several scientific and technological problems have to be solved before one can transfer a biological control method from the laboratory to the field.

First, it is necessary to prove the efficacy and feasibility of the method over several years, under different climatic and cultural conditions. Thus, large amounts of antagonistic inoculum has to be produced. The formulation of this "biopesticide" must be convenient for easy storage, transportation and application in the fields. Such problems are particularly important when agents are applied to control soil-borne plant-pathogens. Usually, the inoculum has to be applied to the soil; thus, large quantities are needed to obtain a homogeneous distribution in the arable layers. It could also be useful to add specific nutrients to allow a better development of the antagonist in the soil.

A strain easily identified and recognizable is helpful, to study the survival of the antagonistic inoculum during all the processes, from its production until it has to be active in soil. Different methods have to be developed to specifically quantify the antagonistic population in the inoculum and in the soil.

Finally, the control method has to be compatible with all other cultural practices and the antagonist itself must be harmless to man and the environment.

Our goal is to describe the steps necessary to achieve biological control of soil-borne plant-pathogens and to illustrate all these aspects by examples, taken from our own experience, dealing with the control of

- take-all of wheat with hypovirulent strains of *Gaeumannomyces graminis* var. *tritici*
- sclerotinia root rot of lettuce with strains of *Trichoderma harzianum*
- fusarium-wilts of tomatoes and carnations with nonpathogenic strains of *Fusarium*.

## 2. Mode of Action of the Antagonists and Efficacy of their Application as Biological Control Agents

### *Mode of Action of the Antagonists*

The three methods developed in our laboratories involved non-pathogenic fungi, as antagonistic microorganism, to control diseases caused by pathogenic soil-borne fungi. But the mode of action of these fungal antagonists differs greatly.

Studies on the natural suppressiveness of some soils to fusarium-wilts showed the role of the native population of nonpathogenic *Fusarium* (Rouxel et al., 1979). Although the mechanisms of competition between pathogenic and nonpathogenic *Fusarium* species are not clearly understood, it was demonstrated that addition of nonpathogenic *Fusarium*, especially *F. oxysporum*, into a conducive soil gave a good control of fusarium wilts of tomatoes and carnations (Couteaudier et al., 1985; Tramier, 1985). The biological control is based on these observations and involves mass-production of non-pathogenic *F. oxysporum* to be incorporated into the soil or the cropping substrate.

Biocontrol of *Sclerotinia* root rot of lettuce can be achieved by application of selected strains of *Trichoderma harzianum* (Davet et al., 1983). The mode of action of this antagonist is complex but, in this case, involves hyperparasitism mostly and antibiosis to a lesser extent. The hyphae of *T. harzianum* colonize the sclerotia of *Sclerotinia minor* and thus contribute to decreasing the density of the pathogen population. The method of control is also based on the mass-production of the antagonist to be incorporated into soil infested with the pathogen.

The well-known phenomenon of take-all decline has been extensively studied, all over the world. Depending on the mechanisms of suppression observed, different methods of biological control have been proposed. Hypovirulent strains of *Gaeumannomyces graminis* var. *tritici* infested by mycoviruses, were isolated in France in 1969 (Lapierre et al., 1970). These strains colonize the roots of wheat and induce defense reactions and other physiological modifications (Lemaire et al., 1979 a and b), with the result that the plant becomes less susceptible to the virulent strain (Tivoli et al., 1974). Moreover the hypovirulence is contagious; virulent strains lose their pathogenicity when they are in contact with the hypovirulent strains (Lemaire et al., 1976). The biological control involves the mass-production of a hypovirulent strain of *G. graminis* and its application as seed treatment.

Table 1. Efficacy of biological control in commercial growing conditions. (1) More than 100 experiments were conducted; results showed the increase in yield after biological treatment of the second wheat crop when take-all was present.

Disease	Antagonist	Results of Biological Control		
		Control % Disease plants	Treated Yield (1)	References
Fusarium wilt of tomatoes	nonpathogenic <i>Fusarium oxysporum</i> and <i>solani</i>	98	20	Couteaudier et al., 1985
Fusarium wilt of carnations	nonpathogenic <i>Fusarium oxysporum</i>	39	2	Tramier, 1985
Sclerotinia root-rot	<i>Trichoderma harzianum</i>	50	23	Davet et al., 1983
Take-all of wheat	Hypovirulent strain of <i>Gaeumannomyces graminis</i>	100	108,7	Lemaire et al., 1977

#### Efficacy of the Biological Control Methods

As soon as they observed an interesting relationship of antagonisms between a microorganism and a pathogenic fungus, plant pathologists try to use the antagonist to control disease. First, they set up experiments in growth chambers and greenhouses. If the results obtained under these conditions are satisfactory, they have to prove the efficacy of the antagonist in normal growing conditions.

Several obstacles have to be overcome. The equipment of the laboratories is usually not sufficient to produce the large amounts of inoculum necessary for field experiments. Scientists do not have all technological facilities that industry possesses. Thus, plant pathologists generally use, on a large scale, the same methods developed to produce small quantities of inoculum, but they do not study all the practical problems posed by mass-production of inoculum.

The data in Table 1 indicate that the proposed biological methods are effective to control diseases in commercial fields or greenhouses, but with the exception of the control of take-all, they are too limited to really prove that these methods can be used on a large scale, in different climatic and soil conditions. More experimentation is needed and, therefore, the problems posed by the mass production of inoculum have to be solved.

### 3. Methods of Mass-Production of Inoculum

At the present time, different procedures have been developed, depending on the local facilities. These examples are discussed below. Few studies have been conducted to compare the efficacy of the different methods available.

#### Mass Production of Hypovirulent Strains of *G. graminis*

The selected strain was isolated in 1969 from a field in French Brittany. It is able to grow and colonize the wheat roots without producing symptoms of the disease and exhibits good stability of its antagonistic properties. Sometimes, in local trials, other hypovirulent strains were better, but on the average it was the most effective strain used in all experiments conducted during the 15 last years. This strain is stored at 4°C on nutrient agar or on autoclaved barley grain.

It grows well in shake culture, in liquid medium with malt enriched with barley seed extract. The optimal temperature is 22–23°C and optimal pH 6.8. A good aeration of the culture is needed but it is not possible to increase the speed of the shaker above 200 rpm to prevent the fragmentation and lysis of the hyphae. When produced in these conditions, most of the hyphae die during the following procedures which precede application to the seed. For this reason, solid state fermentation has been preferred.

Figure 1 shows the method chosen to produce inoculum of the hypovirulent strain of *G. graminis* var. *tritici* and indicates the main conditions at each stage of production and application. Autoclaved barley grain was chosen as a culture medium because inoculum produced had a better survival rate than with other substrates: grown on bran, the fungus died quickly; on straw the inoculum survived for 1 month; produced on barley grain, it was possible to isolate the hypovirulent strain from soil 2 months after its application, and the biological effect on plants was still noticeable after 5 months.

After 3 weeks of culture at 23°C, in the dark, the inoculum is dried at 30°C using a forced ventilation of sterile air. Before use, this inoculum is stored dry at 4°C. To be applied to seeds, inoculum has to be ground using a flourmill. Particles between 100 and 200µm are selected for seed treatment since the smallest particles < 100µm have a low inoculum content and the biggest > 200µm are difficult to use for seed coating.

#### Mass-Production of NonPathogenic *Fusarium*

Two methods for mass production of nonpathogenic *Fusarium* have been developed separately. In Antibes, to control fusarium wilt of carnations,

the non-pathogenic strains of *Fusarium* are grown on a bran-perlite mixture (v/v) amended with malt extract. After 4 weeks at 25°C, the growth medium and the fungus are dried at 35°C during 30 hr and ground to be directly incorporated into the cropping substrate (Tramier et al., 1983).

In Dijon, to control fusarium wilt of tomatoes and muskmelons, the non-pathogenic strains of *Fusarium* are grown in shake-culture on a malt extract. After 10 days, the growth medium is removed by centrifugation and the propagules (mostly conidia), are suspended in sterile water and mixed with talc. The mixture is dried at 18–20°C using dry forced air and then the talc and fungal material is ground in a special mill, sieved through a mesh of 200µm, and stored at room temperature. In these conditions, the fungus survive for more than one year (Tello-Marquina and Alabouvette, 1984). This inoculum is easy to transport and to mix with soil directly as a powder or after having been suspended in water.

These 2 methods seem convenient for biological control, but they have not been yet compared for their respective technological and biological advantages. At the present time, liquid fermentation is the most convenient for mass-production; inoculum is already produced in 400 liters fermentors, but the solid state fermentation may provide a more effective inoculum. Experiments are now in progress to compare the efficacy of different strains of *Fusarium* several formulations, and doses of application.

#### Mass-production of *Trichoderma harzianum*

To produce inoculum of *Trichoderma harzianum* different substrates have been used: autoclaved grain, bran-peat or bran-sawdust mixtures (Wells et al., 1972; Abd el Moity and Shalta, 1981; Elad et al., 1980). But these solid substrates are difficult to sterilize and they form compact balls difficult for the fungus to colonize. Davet et al. (1981) developed an easier method, using non-sterile chopped straw. This substrate is amended with a mineral solution containing allyl alcohol and vinclozolin, which makes the mycelium specific for the growth of *T. harzianum*. This method is well adapted to produce inoculum for microplots experiments but does not allow mass-production.

Recently, great interest has been shown in the use of small pellets of alginate, in which spores of *Trichoderma* can be incorporated (Fravel et al., 1985). However, before using such a technique on a large scale, many problems have to be studied:

- The optimal inoculum concentration per pellet must be determined. Relative cost to produce large quantities of pellets with a low con-

centration of spores or smaller quantities with a high concentration of spores must be determined. From the scientific point of view, it seems more effective to spread a large number of pellets to provide a good distribution of the antagonist in the soil,

- is it useful to add some nutrients to ensure better survival of the fungus in the balls and to provide a specific source of energy useful to its growth into the soil?
- what type of propagule (chlamydo-spores, conidia, mycelium) is best for incorporation in alginate pellets and therefore what type of fermentation should be preferred? In liquid culture *T. harzianum* produces a greater proportion of chlamydo-spores, but the enzyme production is better on solid medium. Depending on the mode of action of the antagonist, one procedure may be preferred to the other.

All these aspects have to be studied with the help of industry, which possesses the technological knowledge for fermentation. It should be kept in mind that a biological method must be beneficial, both for the industry that produces the inoculum and for the farmer who will apply the method.

#### 4. Selective Analysis and Utilization of Marked Strains

Development of biological control methods can be aided by specific techniques to recognize and quantify the population of selected antagonists. To assay the quality of the inoculum, it is necessary to determine the population density in the growth medium, during storage, at the seed surface, in the soil, and on the roots. Quantitative methods using selective media have been developed for *Fusarium* and *Trichoderma*, to follow the evolution of the inoculum density at each stage of its production and application. However, these methods are specific for the fungal species and do not permit recognition of the selected strain of the species. Other procedures have to be used, such as electrophoresis and immunofluorescence. These techniques are being developed in several laboratories to identify the different strains of *T. harzianum* (Zamir and Chet, 1985) and *G. graminis* var *tritici*.

We also hope that, in the near future, techniques of molecular biology will help us to identify the strains of nonpathogenic *Fusarium*. This field of research is being explored, but progress is slow because the problems to be solved are more difficult with filamentous fungi than with bacteria. More cooperation between geneticists and plant pathologists is required.

A biological test is used to detect viable inoculum of hypovirulent *G. graminis* var. *tritici* on seed material. The seeds are germinated in a tube con-



taining wet perlite; 3 weeks later developing roots show the characteristic lesions due to hypovirulent strains. For this fungus, a technique based on immunoenzymatic reactions is also available; results with this technique are comparable with those obtained with the biotest.

Another approach to monitor the survival and development of a specific strain, both on seeds and in the soil, uses marked strains. It is possible to select biotypes resistant to fungicides. U.V. irradiation has been a useful tool to induce mutation and generate new biotypes of *T. harzianum* and *F. oxysporum* resistant to benomyl. These marked strains are not only useful to monitor the survival of the antagonist in the soil, but in an integrated pest management system they could also be more effective than the wild-type antagonist, e.g. they can be used in combination with the fungicide which will give them a competitive advantage over the native microflora.

Genetically marked strains will be necessary to insure proprietary protection of potential biological control procedures.

##### 5. Integration of Biological Control Methods in the Cropping System

To apply a biological control method in a commercial field it must be compatible with all other cultural practices and especially with the common chemical treatments.

This aspect has been extensively studied in the case of seed coating with hypovirulent strain of *G. graminis* var. *tritici*. It has been demonstrated that application of *G. graminis* var. *tritici* is compatible with several chemicals provided the chemicals are applied before the biological treatment. Usual insecticides, such as lindane and antraquinone, have no effect on the fungus; organo-mercuric fungicides, maneb and to a lesser extent oxyne copper, are compatible with *G. graminis* var. *tritici*, but systemic fungicides such as benomyl, thiabendazol and ethirimol rapidly kill the hypovirulent strain of *G. graminis* var. *tritici*. If the use of these fungicides is absolutely necessary selection of strains resistant to them will be required.

In the case of *Sclerotinia* of lettuce, it has been demonstrated that some fungicides may have a synergic effect with *T. harzianum*. Thus the population of sclerotia decreases faster when cyclic imides are applied to the soil previously enriched with *T. harzianum*. *Sclerotinia minor* is susceptible to these fungicides but the strains of *T. harzianum* are tolerant. These observations open the way to an integrated control method (Davet and Martin, 1985).

Other cultural practices determine the efficiency of a biological control method. As mentioned above, efficiency of *T. harzianum* depends on climatic conditions: soil moisture and temperature in particular must be sufficient to favor the development of the fungus. Thus the treatment has to be carried out during spring or early summer and irrigation may be necessary.

To succeed in the control of take-all, by application of a hypovirulent strain of *G. graminis*, it is necessary to observe several conditions. To start a wheat monoculture it is important to sow an inoculated wheat seed before the disease has become severe. Usually the treatment is applied with the second culture, but it may be necessary to protect the first one, and it is not recommended to sow with an inoculated wheat after a spring-wheat crop. It is also important to remove the straw of the previous culture and to seed after tillage, early in autumn. One of the main conditions for success is to choose a wheat variety which shows the best response to inoculation with the hypovirulent strain. A wide genetic variability exists that is being used to create new cultivars well adapted for biological control (Lemaire et al., 1982). Finally the fertilization has to be optimum, because deficiencies in mineral nutrition, especially of nitrogen, limit the defense reactions of the plant.

The success of biological control methods based on soil application of an antagonistic microorganism also depends on the receptivity of soils to this antagonist. Soils show different levels of suppressiveness to soil-borne plant-pathogens (Alabouvette et al., 1982), and therefore can favor or limit the development and the expression of the antagonistic capacities of a biological control agent. This has been demonstrated in the case of nonpathogenic strains of *F. oxysporum* used to control fusarium wilts (Corman et al., 1986).

At the present time, to avoid this problem in horticultural and vegetable crop production, biological control methods are applied in highly conducive cropping substrates or in fumigated soils. In soilless cultures the substrates used for the first time are conducive to the nonpathogenic strains, which occupy the space and compete with subsequent infestation of the pathogenic strain. In tomato crops under greenhouses, fumigation with methylbromide is necessary to control corky-root due to *Pyrenochaeta lycopersici*. This fumigation destroys a large proportion of the soil microflora, including the pathogenic *Fusarium* and allows colonization of the rooting medium by the nonpathogenic *Fusarium* added just after the fumigation.

More research is needed in this field to better understand the mechanisms of soil receptivity to antagonistic microorganisms and to learn how to make

a soil conducive for a biological treatment.

## 6. Other Problems that Limit Commercial Application of Biological Control Methods

The biological control methods proposed have been developed in the laboratories of I.N.R.A. but have not yet been used at a commercial scale. The remaining problems justify cooperative studies with other scientists, industry and extension services, because they can not be solved by plant pathologists alone.

To prove the efficacy of the methods in large-scale field experiments in several localities, under different climatic and soil conditions, over a period of several years, it is necessary to produce large amounts of inoculum and to create a network for experimentation. This first problem has been overcome for take-all control during 1979 and 1980, when a small production unit of I.N.R.A. produced enough inoculum (several hundred kilos) to treat seeds necessary for more than 100 ha of wheat. A large program of experimentation concerning the application of nonpathogenic *Fusarium* to control fusarium wilts of tomatoes and carnations is in progress. This program is supported by a grant of the National Agency for Valorisation of Research (ANVAR), but presently, no support has been forthcoming from industry, which seems to be sceptical about the interest in biological control methods and believe that the potential market will be too small for sufficient profit. As mentioned above, the technological knowledge of industry is needed to compare the different methods of inoculum production, formulation and to determine the production costs of these methods.

To apply a biological method in the field, it is necessary to prove the harmlessness for man and the environment. At the present time in Europe, there are no specific laws for biological control agents, which have to satisfy the same tests as a chemical product, however these tests are not adapted for microorganisms. Toxicological studies have been conducted to prove that the hypovirulent strain of *G. graminis* var. *tritici* is not toxic to animals either directly or indirectly, since it does not produce any mycotoxine, but it is difficult for a biological product to satisfy the same criteria of stability demanded for a chemical molecule. The long term effects are difficult to study, and at present no one can assert that a nonpathogenic strain of *Fusarium* or a hypoaggressive strain of *G. graminis* var. *tritici* will never be able to acquire pathogenicity. Basic research on the genetical mechanism of the pathogenicity is needed, but it seems ridiculous to delay the application

of any biological control method until the mechanism of pathogenicity has been clearly understood!

Depending on the example taken, it is sometimes difficult, but always possible, to estimate the potential market for these biopesticides, and with the help of industry, to estimate the cost of the product. As noted, biopesticides need to be stored and delivered differently than chemical pesticides. Their survival is limited and depends on the conditions of storage, especially moisture and temperature. Their application may need some changes in the usual cultural techniques; thus education of the farmer is also needed. Farmers must not expect the same results with biological control as with chemical control: the aim is not the total suppression of the disease but rather to maintain a satisfactory level of yield in the presence of the pathogen. Environment is of greater importance with biological than with chemical control and its influence cannot be known without large scale experimentation over several years. Biological control methods possess some inconveniences but also great advantages. They are non-toxic so that the plant products are of better quality, which is a good argument especially for customers buying vegetables and fruits. Biological control is more specific than usual chemical treatments, thus it preserves the natural balance of the environment. In some cases, biological control methods will be necessary as pathogenic fungi become tolerant or resistant to the fungicides in use. A good example is the extensive use of biological control of insects in vegetable crops, under greenhouse conditions.

## 7. Conclusions

In the past, mass introduction of a biological agent into the soil was largely criticized based on the knowledge that soil is microbiologically buffered and will present a strong resistance to the introduced microorganism. Despite the validity of this concept, well illustrated by studies dealing with soil suppressiveness to soil-borne plant-pathogens, recent results show that some possibilities exist to modify, at least temporarily, the soil microbial balance to suppress pathogens. Many microorganisms, having various modes of action can potentially be used as biological control agents. In spite of extensive studies in laboratories, only a few of these agents have been tested in field experiments. One reason is the lack of knowledge in the field of mass-production and formulation of the antagonistic inoculum. It is true that basic research is still needed, e.g. to better understand the mechanisms of soil-suppressiveness to an introduced microorganism, to better know the ecological requirements of the proposed biological agents and to select or create

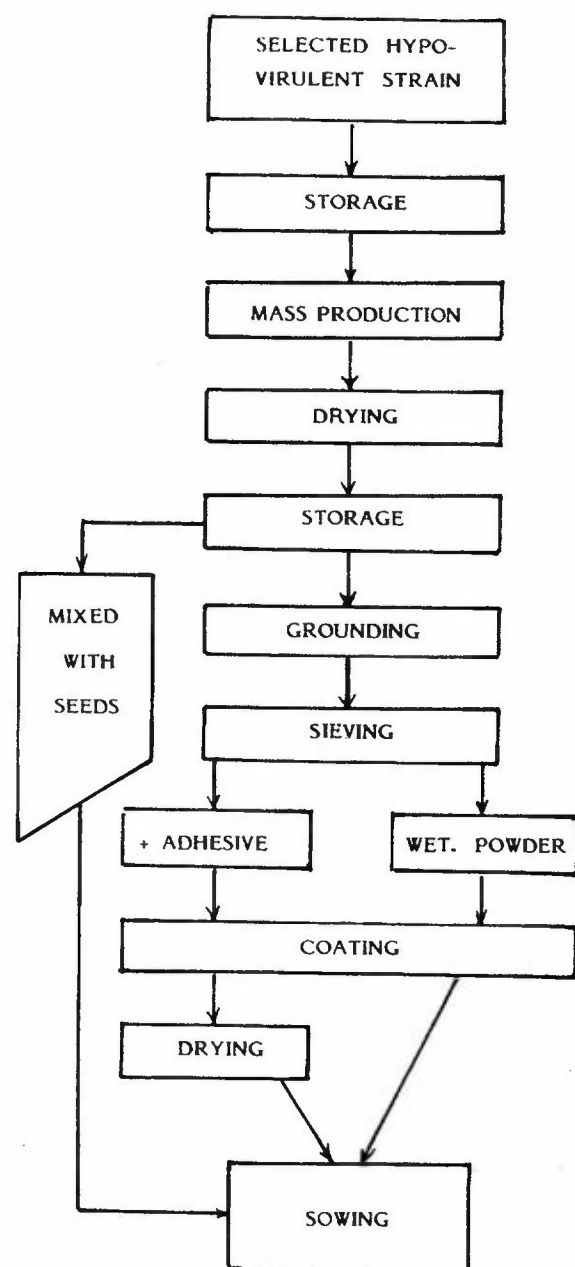


Figure 1. Processes for mass-production and application of hypovirulent strain of *Gaeumannomyces graminis* var. *tritici*.

new biotypes adapted to integrated pest management, but we believe that more research is needed the most on the technological aspects of the mass-production and delivery procedures. Already it has been proven that the efficacy of a biological agent depends on the growth medium, and the formulation, including or excluding a specific food base. We need to compare on a large scale the efficacy of different formulations of inoculum resulting for standardized production procedures. These studies will not be done by plant pathologists alone. They require the participation of industry, which has the technological knowledge, and of the extension services and farmers, to set up a large network of experimentation.

We also have to investigate the side-effects on man and environment of large scale application of biopesticides, and to study the stability of strains used. Additionally we also need from the the regulatory agencies specific guidelines that clearly recognize the biological differences and the special circumstances of products compared with chemicals.

Finally, development of biological control of disease requires a change in the thought processes of manufactures and farmers, who do not believe in the chance of biological control. As the traditional methods of control show their limitations (resistance of the pathogens to pesticides, pollution of environment, toxic residues in food), and as the biological methods show their effectiveness in controlling pests or improving the nitrogen fixation of Leguminous crops, we have to be optimistic and we hope that progress will be made in the near future in the field of biological control of soil-borne plant pathogens.

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