Identifying Microorganisms Involved in Specific Pathogen Suppression in Soil

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Abstract

Suppressive soils hold considerable potential for managing soilborne pathogens. When the suppressiveness has a biological origin, identifying the causal organisms is the crucial step in realizing this potential. Armed with such knowledge, it may be possible to develop effective and sustainable pest management strategies through application of these organisms or agronomic practices that influence their population densities. This chapter focuses on the development and utilization of a population-based approach for identifying microorganisms involved in specific pathogen suppression. Key experimental design principles of the approach are explored by examining experiments characterizing the biological nature of take-all decline. We also describe how this approach was used to identify microorganisms that suppress the sugarbeet cyst nematode. Additional experimental design considerations and future directions for such investigations are also discussed.

INTRODUCTION

Optimal farming decisions should involve an analysis of a wide range of variables. Agronomic parameters can include crop species and cultivar, crop rotation sequence, and tillage practices. Environmental factors might include soil physicochemical characteristics as well as climatic variables such as rainfall, humidity, and temperature. Other parameters could include the use of agrochemicals such as pesticides, growth regulators, and fertilizers.

Microorganisms are another variable that should be considered in farming decisions. The potential damage caused by plant pathogens can have a considerable effect on the selection of crop cultivars and rootstocks, crop rotations, planting density and timing, seed treatments, and agrochemicals. On the other hand, a grower might choose to utilize beneficial microorganisms via seed treatments or soil amendments. For example, rhizobia and mycorrhizae may increase plant productivity (105, 106) while other microorganisms can enhance plant defenses (17, 100). Another relatively unexploited yet promising resource for pathogen control may come from understanding suppressive soils.

Pathogen-suppressive soils have been defined as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (20, p. 254). This definition, of course, presumes the conditions of the normal disease triangle: the presence of a virulent pathogen, a susceptible host, and otherwise disease-conducive environmental conditions. Whereas general suppression refers to the inhibition of pathogens resulting from the total amount of microbial activity, specific suppression refers to antagonistic activity against the pathogen by individual microorganisms or a more narrowly defined group of organisms (20, pp. 255–56). One key in realizing the potential of specific suppression for disease management is to identify the causal microorganisms and their activity requirements. Armed with such knowledge, it may be possible to create new strategies for disease management by transferring the organisms to conducive soils. In addition, through the development of assays to track these organisms, agronomic practices that influence the development and maintenance of suppressiveness could be identified. Finally, by enumerating the densities of both the pathogen and suppressive organisms, it may be possible to develop more effective assays to predict disease potential.

Investigations endeavoring to identify microorganisms involved in specific suppression must overcome several obstacles. First, the number of species inhabiting soil is often very large, with estimates ranging from thousands to millions (35, 97). Second, most microorganisms are not readily cultured by standard techniques (3). Finally, even if detailed descriptions of the microorganisms inhabiting an environment can be obtained, how are the suppressive organism(s) distinguished from the multitude of other organisms? Or, as we like to think about these investigations, how do we find the needles in the haystack?

We focus on the development and utilization of population-based investigations for identifying microorganisms involved in specific suppression. What follows is a description of a population-based approach. We then use examples to explore key experimental design principles of the approach. Finally, additional experimental design considerations and future directions for such investigations are discussed.

POPULATION-BASED APPROACH

Several approaches have been developed to identify microorganisms involved in specific in situ processes. They include applications of isotopes (13, 74) and bromodeoxyuridine (9) to identify microorganisms that utilize specific substrates, rRNA-based methods to identify metabolically active organisms (29, 40, 58,

90), and genomic DNA analyses (6, 101). Another approach is to correlate the abundance of microbial taxa with levels of the in situ process; we refer to this later general strategy as a population-based approach.

Population-based investigations of suppressive soils can be performed by examining the microbial community compositions in soils possessing various levels of suppressiveness. Microbial communities can be examined using a variety of culture or culture-independent methods. Different levels of suppressiveness can be created by manipulating the microbial communities with physical, chemical, and biological methods such as heat treatments, antimicrobial agents, and nutritional or microbial amendments. Alternatively, naturally occurring soils with different levels of suppressiveness can also be utilized. The relative abundance of each taxon can then be associated with levels of suppressiveness. Taxa exhibiting the strongest correlations will represent organisms putatively involved in the suppressiveness. This general approach is illustrated in **Figure 1**. In this example, there are three soils with different levels of suppressiveness. Except for *Bacillus*, the population densities of all of the taxa are the same in each soil. Because the population densities of *Bacillus* positively correlate with suppressiveness levels, this suggests that it may be involved in this process, and it would therefore become a candidate for further study.

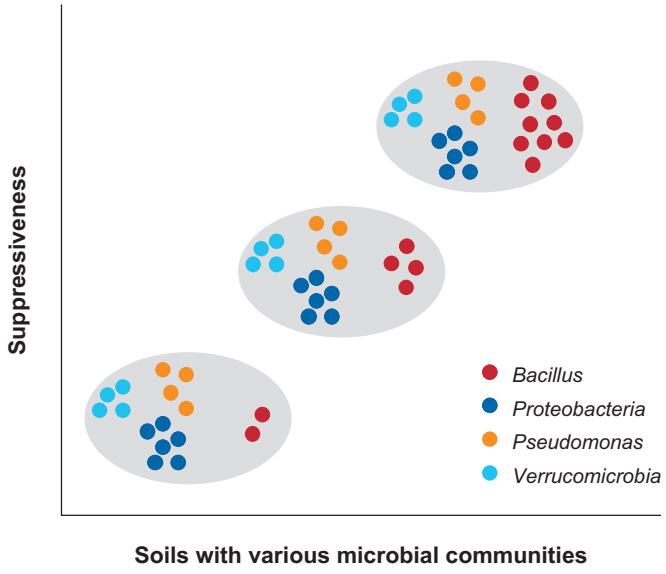

Figure 1

Illustration of the principle of the population-based approach. To identify microorganisms involved in specific pathogen suppression, microbial community analyses are performed on soils exhibiting various levels of suppressiveness. The relative abundance of each taxon is associated with levels of suppressiveness. Taxa exhibiting positive correlations represent organisms putatively involved in the suppressiveness.

Experimental Design Principles of the Population-Based Approach

The effectiveness of the population-based approach can be enhanced by following several experimental design principles. Below we describe selected investigations to demonstrate these principles, which include (*a*) identifying or creating soils with various levels of suppressiveness, (*b*) examining sites where the suppressive organisms function, and (*c*) utilizing an appropriate method for microbial community analysis. Examining soils with different levels of suppressiveness enables identification of organisms whose population densities correlate with levels of the suppressiveness. Identifying the site where the antagonistic action takes place increases the likelihood of identifying the suppressive organisms, because it can narrow the focus of the investigations. For example, if the suppressive organisms are known to influence a particular life-cycle-stage of a pathogen, then the microbial community analysis should be performed on the pathogen in that stage. This can eliminate the need to examine environments with much more complex microbial communities such as those found in soil. Finally, utilizing a method that allows analysis of microbial community composition at the necessary taxonomic resolution and level of coverage is another factor important to the likelihood of success.

TAKE-ALL DECLINE

Take-all is an important root disease of wheat caused by the fungus *Gaeumannomyces*

TAD: take-all decline

graminis var. *tritici*. Take-all decline (TAD) is "the spontaneous decrease in the incidence and severity of take-all that occurs with monoculture of wheat or other susceptible host crops after one or more severe outbreaks of the disease" (107). The first descriptions of TAD were reported in the 1930s (34, 41). A considerable body of experimental evidence supports a microbial causation for this pathogen suppressiveness. Below we examine and discuss experiments that characterize the microbiological nature of TAD in relation to the experimental design principles of the population-based approach. Although some of the earlier reports refer to the take-all fungus by its former name *Ophiobolus graminis*, we use its current name *G. graminis* throughout for consistency.

Identifying or Creating Various Levels of Suppressiveness

Different levels of suppressiveness can be identified or created by (*a*) following the development of the suppressiveness over time, (*b*) examining naturally occurring suppressive soils, and (*c*) using various soil treatments.

Development of suppression over time. In greenhouse trials, Gerlagh monitored the levels of TAD that developed through four crop cycles and inoculation with different strains of *G. graminis* (39). Levels of suppressiveness increased with the number of crop cycles. In addition, development of suppressiveness depended on the presence and virulence of *G. graminis*. These results are consistent with the idea that events associated with severe disease lead to a buildup of microorganisms that cause a decline in the disease.

These results also demonstrate the potential utility of temporal analyses for population-based studies endeavoring to identify microorganisms involved in specific suppressiveness. Such studies could be accomplished by monitoring the population densities of microorganisms throughout the development of suppressiveness. Microorganisms putatively involved in suppressiveness will be those whose abundance positively correlates with levels of the suppressiveness. To provide added statistical power in these correlation studies, additional levels of suppressiveness could be created by using strains of *G. graminis* with various levels of virulency. In addition, similar experiments also could be performed by monitoring microbial populations over time in suppressive soils planted with crops that reduce or eliminate the suppressiveness.

Naturally occurring suppressive soils. Several studies have examined root-associated bacteria from naturally occurring soils exhibiting various levels of TAD. Greater percentages of *Pseudomonas* strains exhibiting in vitro antagonism to *G. graminis* were obtained from suppressive than from conducive soils (20, p. 257; 86, 87). In addition, greater percentages of *Pseudomonas* strains with the ability to inhibit *G. graminis* root lesions were obtained from suppressive than from conducive soils (108).

Sarniguet & Lucas (79) described a related phenomenon with *Gaeumannomyces graminis* var. *avenae* (take-all) on turfgrass (*Festuca* sp.). Here, over time, patches of take-all-damaged turfgrass were recolonized by healthy grass. The authors suggest this phenomenon is similar to TAD in wheat, where disease outbreaks can lead to a decrease in the severity of the disease. Examination of bacteria from different regions of these turfgrass patches showed that a greater percentage of strains exhibiting in vitro antagonism to *G. graminis* were obtained from regions where the disease had occurred compared to areas where the disease was currently damaging the grass or where it had not occurred.

These results demonstrate the utility of examining naturally suppressive soils when attempting to identify microorganisms involved in specific suppressiveness. By examining the microbial communities in soils with different levels of suppressiveness, organisms whose abundance positively correlates with levels

of suppressiveness can be identified. However, because such studies are frequently performed with soils from different locations, and that possess different physicochemical characteristics, one potential problem with this approach is that many of the differences in microbial communities among the soils may be associated with differences in abiotic soil characteristics, and not with the suppressiveness. In cases like the turfgrass example, where various levels of suppressiveness develop in the same soil type, suppressive organisms may be easier to identify because differences in the microbial communities will be more likely associated with the suppressiveness.

Soil treatments. To determine the microbiological nature of TAD, Gerlagh examined the effect of a variety of soil treatments on the pathogen suppressiveness (39). These treatments included autoclaving, biocidal soil fumigants such as chloropicrin and methyl bromide, as well as exposure to different temperatures. The biocidal treatments had varying effects on the suppressiveness; some of them were dose dependent. Soil treatments at various temperatures suggested that the suppressive organisms were eliminated at approximately 60°C. These investigations provided convincing evidence for a biological cause of TAD. In addition, because of the taxa-selectivity of some of the soil treatments, these experiments also allowed inferences to be made concerning the types of microorganisms that cause the disease decline.

The taxa-selectivity of the treatments also created a series of soils possessing various levels of suppressiveness. As described above, analyzing the population densities of microorganisms in such soils can lead to the identification of organisms that correlate with the suppressiveness. In addition, studies utilizing soil treatments have the aforementioned advantage of comparing the microbial communities in soils with similar physicochemical characteristics.

Examining Sites where Suppressive Microorganisms Function

To increase the likelihood of identifying microorganisms involved in pathogen suppressiveness, it is useful to determine where the suppressive microorganisms function. In TAD studies, microscopic observations suggested that lysis of *G. graminis* hyphae was associated with bacterial colonization (75, 103). A greater percentage of bacterial isolates exhibiting in vitro antagonism to *G. graminis* was obtained from take-all-infected than noninfected plant material (103). In addition, a greater percentage of bacteria exhibiting in vitro antagonism to *G. graminis* were isolated from rhizoplane than rhizosphere samples (86). These observations and results suggest that *G. graminis*-antagonistic organisms may be associated with the take-all fungus or take-all-infected sites on plant roots. Such information can be used to increase the probability of identifying the suppressive organisms, as it indicates that the microbial community analyses could be performed on samples from these specific sites instead of on the more complex soil environment.

Utilizing an Appropriate Method for Microbial Community Analysis

Identifying the appropriate method(s) for analyzing microbial community composition can be a considerable challenge. Because of the complexity of soil microbial communities and the difficulties of culturing most of these organisms, there are typically no methods that can generate the preferred levels of taxonomic resolution and coverage in a cost-effective and timely manner. However, developments in both culture and culture-independent experiments have led to improvements in these capabilities (see Additional Experimental Design Considerations and Future Directions section below). For example, in TAD studies, strains of *Pseudomonas* were shown to exhibit different abilities to colonize wheat roots and suppress take-all disease (73, 83). By applying

molecular genotyping methods, investigators were able to develop a framework to characterize and differentiate these strains (54, 62, 64, 65, 73, 83). The ability to identify strains with beneficial properties and to track them in their natural environment should lead to a greater understanding of their roles in TAD, improvements in strain selection, and the development of better disease management strategies.

A NEMATODE-SUPPRESSIVE SOIL

To further explore the population-based approach, the following section examines experiments to characterize a nematode-suppressive soil. First, the history and development of the suppressiveness are described. We then examine experiments that demonstrated the biological nature of the suppression as well as culture and microscopic investigations of fungal colonization and parasitism of nematode cysts and eggs. Finally, culture-independent experiments to identify microorganisms involved in the suppressiveness are examined and discussed in relation to the experimental design principles of the population-based approach.

History and Development of *Heterodera schachtii* Suppression in Field 9E Soil

Field 9E has been the focus of a decade-long investigation into the cause and nature of a nematode-suppressive soil. This particular field, an approximately 1-hectare site at the University of California-Riverside's Agricultural Experiment Station, was amended in 1975 with two truckloads of soil obtained from a sugarbeet field located in a neighboring city. The soil, which was heavily infested with the sugarbeet cyst nematode (*Heterodera schachtii*), was spread uniformly across field 9E. The purpose of the amendment was to establish the nematode so that the field site could be used for efficacy testing of newly developed nematicides. During the following years, cropping at this site consisted predominantly of hosts of *H. schachtii*. As intended, the increase in nematode populations was accompanied by severe disease incidence resulting in significant yield reductions during the first few years (I. Thomason, personal communication). However, in subsequent years, population densities of *H. schachtii* and disease incidence rapidly declined, making the field unsuitable for the nematicide efficacy trials. Although it was hypothesized that microorganisms antagonistic to *H. schachtii* caused the population decline, this was not demonstrated for nearly two decades.

Biological Nature of the Suppression

A series of experiments characterized the biological nature of the nematode suppressiveness. 9E soil was exposed to various treatments, infested with laboratory-reared *H. schachtii*, and planted with a host crop. Soil treatments included fumigants such as methyl bromide, methyl iodide, or metam sodium as well as 30-min exposure to aerated steam (109). When *H. schachtii* was reintroduced and presented with a host, the population densities of the nematode increased in most of the treated soils but remained low in the untreated control soil. In greenhouse trials, amendment of 0.1% 9E soil to fumigated 9E soil transferred the suppressiveness to the conducive soil, whereas a 1% amendment was sufficient to achieve a similar effect in a fumigated field site (110). Efficacy of the transfer and subsequent suppression followed a typical dose response: the more 9E soil transferred, the stronger the suppressive effect on the nematode populations. Exposure of 9E soil to 55°C for 30 min reduced the suppressiveness to the level observed in fumigated soil (111). In addition, cysts of *H. schachtii* that developed in the suppressive soil transferred the suppressiveness to fumigation-induced, conducive 9E soil (111).

Population studies of *H. schachtii* in the 9E soil revealed that the nematode

suppressiveness became evident in the second nematode generation (37, 110, 111). The number of females as well as males increased notably in the conducive soil, whereas they did not change in the suppressive soil. In addition, the reduction of males was larger than the reduction of females (37). This suggested an additional antagonistic effect that specifically or more effectively impaired developing males, since the observed decline in infective juveniles should have resulted in similar reductions of both males and females. The elimination of males might be an important factor of this unusually effective suppressiveness because reproduction of *H. schachtii* requires functional males.

Culture and Microscopic Investigations of the *H. schachtii* Suppressive Soil

Culture and microscopic analyses of *H. schachtii* cysts and eggs from the 9E soil revealed considerable fungal colonization and parasitism. Nematode-destroying fungi such as *Dactylella oviparasitica*, *Fusarium oxysporum*, other *Fusarium* spp. and *Paecilomyces lilacinus*, as well as other unidentified fungi were isolated on agar media (111). Microscopic examination of eggs of *H. schachtii* from 9E soil frequently found them parasitized with internal hyphae and chlamydospores. More specifically, we observed *F. oxysporum* macroconidia in nematode eggs, which also exhibited a faint orange-reddish color, presumably due to metabolites of this fungus (36). The loss of suppressiveness after exposure of 9E soil to 55°C also coincided with a reduction of *Fusarium* propagules below the detection level on Komada's medium (111).

Although these investigations identified numerous fungi, many of which parasitized *H. schachtii*, they did not reveal the extent to which each fungus contributed to the suppressiveness. In the next set of investigations, we attempted to obtain this information by determining how the population densities of the fungi related to the levels of *H. schachtii* suppressiveness.

Population-Based Approach to Identify Microorganisms Involved in *H. schachtii* Suppression

To identify microorganisms involved in the 9E suppressiveness, we used a population-based approach comprised of three phases: (*a*) identifying rRNA genes whose abundance correlates with levels of *H. schachtii* suppressiveness, (*b*) validating the phase I population trends with sequence-selective quantitative PCR (qPCR), and (*c*) validating the function of the organisms by reintroducing them into soil. This experimental approach has several key features. The first important feature was the utilization of several different methods for creating various levels (or gradients) of *H. schachtii* suppressiveness. By identifying those rRNA genes whose abundance consistently correlated with levels of *H. schachtii* suppressiveness (and which were produced by several different methods), the number of putatively causal organisms was reduced, simplifying the reintroduction studies. The other important features were the two validation components. The qPCR experiments provided both quantitative measurements for and an independent validation of the phase I correlation studies. The phase III experiments provided a more definitive validation by examining the ability of specific microorganism(s) to suppress *H. schachtii* after they were reintroduced into soil.

Examining sites where the suppressive organisms function. The microbial community analyses were performed on *H. schachtii* cysts isolated from the suppressive soils, instead of the soils themselves. We targeted the cysts for two reasons. First, microscopic investigations showed that fungi colonized and parasitized *H. schachtii* cysts and eggs, indicating that the beneficial organisms may be contributing to the suppressiveness through parasitism that either reduced

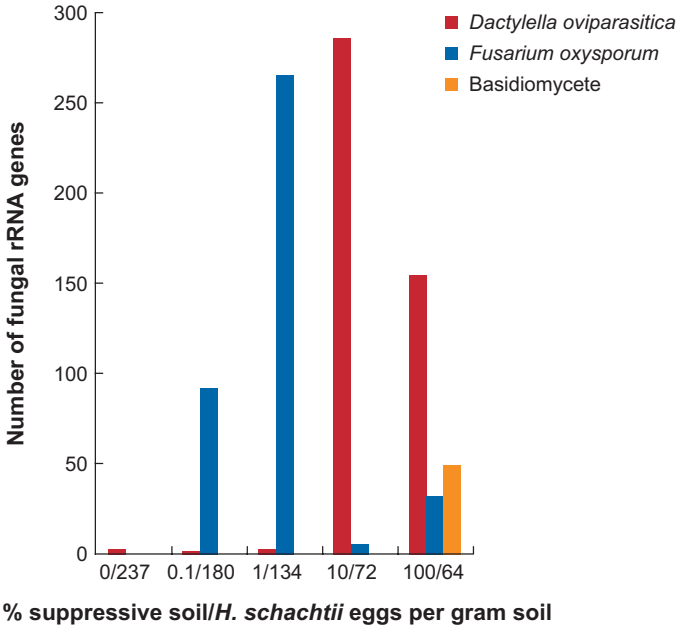

Figure 2

Abundance of the fungal rRNA genes in cysts of *H. schachtii* from soils possessing various levels of suppressiveness to *H. schachtii*. Different levels of suppressiveness were produced by mixing various amounts of suppressive 9E and fumigated 9E soil. Population densities of *H. schachtii* (eggs per gram soil) indicate the level of suppressiveness. This figure is an adaptation of tables 1 and 4 from Yin et al. (114).

reproduction or egg hatch. Second, and perhaps more important, cysts from the 9E soil exhibited the ability to transfer the suppressiveness to conducive soil (111, 113, 114). This result provided convincing evidence that the organisms primarily responsible for the suppression inhabited the cysts, directing the following population studies to be performed on the cysts.

Creating various levels of *H. schachtii* suppressiveness. In phase I of this project, various levels of suppressiveness were created by mixing different amounts of suppressive and fumigation-induced conducive soil (114). These soil mixtures were planted with Swiss chard and then infested with juveniles of *H. schachtii* (J2) four weeks later. Approximately two nematode generations after infestation, population densities of *H. schachtii* were measured. The treatments containing 100% and 10% suppressive soil produced the lowest numbers of cysts and eggs of *H. schachtii*, whereas the treatments containing 1%, 0.1%, and 0% suppressive soil produced successively higher numbers of nematode cysts and eggs, although some of these values were not significantly different.

OFRG: oligonucleotide fingerprinting of ribosomal RNA genes

Identifying fungi from soils possessing various levels of *H. schachtii* suppressiveness. To identify fungi involved in the suppressiveness, an rRNA gene analysis was performed on *H. schachtii* cysts from soils comprised of different amounts of suppressive and conducive soil (described above). The rRNA gene analysis was performed by using oligonucleotide fingerprinting of rRNA genes (OFRG), which is an array-based method that allows analysis of specific taxonomic groups including bacteria and fungi (98, 99).

Three predominant fungal phylotypes were identified by the OFRG analysis (114). In the treatments containing 100% and 10% suppressive soil, the most abundant phylotype had high sequence identity to rRNA genes from various nematode-destroying fungi (**Figure 2**); subsequent nucleotide sequence analysis of rRNA genes from fungal strains in our culture collection showed that the fungus represented by this phylotype was most closely related to *D. oviparasitica*, which was originally described by Stirling & Mankau (92). In the 1% and 0.1% suppressive soil treatments, the most abundant phylotype had high sequence identity to rRNA genes from *F. oxysporum*. The third major phylotype was found only in the 100% suppressive soil treatment, and was related to several genera in the *Basidiomycota*. These data corroborated the culture-based experiments, which showed that *D. oviparasitica* and *F. oxysporum* were common colonizers of *H. schachtii* cysts and eggs (111). However, the relative abundance of the fungal rRNA genes derived from the different soil treatments indicated that *D. oviparasitica* was the key component of the nematode suppressiveness.

In the phase II studies, sequence-selective PCR assays were used to further examine the associations between the abundance of the fungal sequences and the levels of *H. schachtii* suppressiveness (114). Experiments examining the three predominant fungal sequences corroborated the population trends detected by the OFRG analysis. These PCR assays were then used to examine cysts from soils possessing various levels of *H. schachtii* suppressiveness, but which were produced by four biocidal soil treatments and by transferring cysts from 9E and fumigated 9E soil into fumigation-induced, conducive 9E soil. For the biocidal treatments, the largest amounts of *D. oviparasitica* PCR product came from the soils possessing the highest *H. schachtii* suppressiveness. For *F. oxysporum*, the largest amount of PCR product came from the soil with the lowest *H. schachtii* suppressiveness while the smallest amount of PCR product came from the soil with the highest suppressiveness. For the basidiomycete, PCR products were only observed in one of the highly suppressive soils. For the cyst transfer experiment, greater amounts of all three fungal sequences were detected in soils amended with cysts that developed in the 9E soil than with cysts that developed in fumigated 9E soil. Finally, all three fungal sequences were detected in 9E soil.

These results demonstrate the value of examining soils with more than two levels of suppressiveness, and which are produced by several different methods. For example, when the sequence-selective PCR assays were used to examine the cyst transfer experiment, which created two levels of suppressiveness, all three fungal rRNA genes were detected in higher amounts in cysts derived from the suppressive than from conducive soil. However, for both the biocidal and the soil mixture experiments, where more than two levels of suppressiveness were created, it became clear that *D. oviparasitica* was the most likely causal agent, as its rRNA genes were the only ones that consistently correlated with high levels of suppressiveness. Conversely, when cysts from both of these methods were examined with the sequence-selective assay for the basidiomycete, its abundance did not consistently correlate with high levels of suppressiveness. Given the amount of time and labor involved in both isolating microorganisms from soil and performing reintroduction experiments, we suggest that the general strategy of reducing the number of putatively causal organisms by examining only those organisms that consistently correlate with various levels of suppressiveness (and which are produced by different methods) will be an important experimental design element for investigations of other suppressive soils.

Fungal reintroduction studies. In phase III of this project, strains of *D. oviparasitica* and *F. oxysporum* were examined by adding them to fumigation-induced, conducive 9E soil in greenhouse trials (70). As previously described, rRNA gene levels of *F. oxysporum* were highest in cysts from soils with minimal to modest levels of suppressiveness (**Figure 2**). When added to conducive soil, *F. oxysporum* consistently reduced population densities of *H. schachtii* compared to the control, although these differences were not significant. On the other hand, the abundance of rRNA genes of *D. oviparasitica* was found to positively correlate with high levels of suppressiveness (**Figure 2**). When added to conducive soil, *D. oviparasitica* produced the same high levels of suppressiveness as the naturally suppressive 9E soil. These results support the underlying principle of the population-based approach: microorganisms involved in suppressiveness can be identified by correlating their abundance with levels of the suppressiveness.

D. oviparasitica was examined further by assessing its ability to reduce population densities of *H. schachtii* in longer-term trials and in different soil types. A single application of *D. oviparasitica* to fumigated 9E soil produced stable suppressiveness to *H. schachtii* over two cropping cycles and at least six nematode generations in combined field microplot and greenhouse studies (69). When added to

four fumigated agricultural soils, which possessed different physicochemical characteristics, *D. oviparasitica* reduced population densities of *H. schachtii* in all four soils (68). When *D. oviparasitica* was added to nonfumigated portions of the same four soils, it reduced population densities of *H. schachtii* in the soils that did not exhibit preexisting levels of suppressiveness. We suggest that *D. oviparasitica*'s ability to reduce populations of *H. schachtii* in different soil types may be associated with its ability to colonize plant roots and to infect late developmental stages (4th stage juveniles and adult females) of the nematode and its eggs (37, 68, 69).

ADDITIONAL EXPERIMENTAL DESIGN CONSIDERATIONS AND FUTURE DIRECTIONS

Several factors can influence the likelihood of success when utilizing a population-based approach. First and foremost, the population densities of the beneficial microorganisms (or the marker used to track them) must covary with levels of the suppressiveness. Unfortunately, such relationships are not likely to be known prior to initiating the experiments. However, if one assumes that these relationships exist, then the experimental design and the methods used for these investigations should be optimized to increase the likelihood that the correlations are detected. Below we discuss additional experimental design considerations and future directions for such studies.

Creating or Identifying Samples with Various Levels of Suppressiveness

As described above, one important experimental design element is to examine several sets of soils possessing various levels of suppressiveness. By focusing the subsequent validation studies on those microorganisms that correlate in all experiments, this should narrow the focus of the investigations. Given the complexity of the microbial communities in soil, this will likely be an important experimental design consideration. The *H. schachtii* studies described in this chapter demonstrated the utility of this design element, as it facilitated the identification of the fungus that appears to be causing the nematode suppressiveness in the 9E field soil.

A variety of methods for creating samples with various levels of suppressiveness have been developed, several of which have been presented above. Below we describe additional methods and experimental design considerations.

Development of suppression over time. Examining temporally associated shifts in microbial populations can be a useful strategy for identifying microorganisms involved in specific suppressiveness. As described above, levels of specific suppressiveness can develop or be eliminated by several factors including crop species and pathogen virulence, among others. Identifying those organisms whose population densities correlate with such shifts can lead to the identification the causal organisms.

It is also possible that following shifts in microbial populations associated with the development of suppressiveness will be essential in understanding this process. For example, specific microorganisms may contribute to events that initiate the development of specific suppressiveness, but these organisms may not be present when the suppressiveness is fully developed. Important successional events such as this are common in plant pathology, as the more specialized pathogen is eventually replaced by nonpathogenic, saprotrophic microorganisms (20, p. 64). In a related phenomenon, application of the biological control agent *Bacillus cereus* UW85 led to altered rhizosphere bacterial communities, even though the introduced organism was no longer an abundant member of the community (39a). [For additional examples of temporal shifts in microbial communities associated with specific processes see (31, 42, 43, 45, 56, 66, 72, 80, 93).]

Soil treatments. There are numerous soil treatments that alter microbial community composition, and therefore can be used to create soils with various levels of specific suppressiveness. Such methods include (*a*) selective antimicrobial agents (1, 25, 52, 55, 94, 113, 114); (*b*) broad-spectrum soil fumigants such as methyl bromide (39, 84), chloropicrin (39), methyl iodide (109) or metam sodium (109); (*c*) gamma-radiation (1, 52); (*d*) nutritional amendments (20); and (*e*) manipulation of environmental parameters such as water potential (19), pH (81, 88), soil aeration (20), and temperature (1, 14, 39, 81, 84, 111). In fumigant studies, microbial communities can be differentially manipulated by utilizing fumigants with various selectivities and/or at different rates (46) or exposure times. Additional gradients can be created by utilizing any one of the above treatments, and then mixing the treated sample with various portions of a sterilized sample. This approach can produce a series of samples with different microbial communities (113, 114), because microorganisms are being added to samples possessing a variety of nutrient levels (dead organisms) and unoccupied niches.

Examining Sites where the Suppressive Organisms Function

Targeting investigations toward important sites and/or performing experiments at scales relevant to the organisms under investigation can also facilitate the identification of microorganisms involved in specific suppressiveness. For example, to identify soil microorganisms that influence or are influenced by plant roots, investigations should target root-associated organisms instead of those in the soil. However, such investigations can be technically challenging, as it is difficult to precisely separate rhizosphere and bulk soil. With the development of new methods such as laser capture microdissection (33), it may be possible to more precisely sample locations important to microbial studies. [For examples where spatial heterogeneity of microbial populations may be associated with functional processes see (15, 45, 56, 66, 67, 95).]

Utilizing an Appropriate Method for Microbial Community Analysis

Population-based studies require a method for analyzing microbial community composition that possesses several important attributes. Given the complexity of most microbial communities and the need to examine numerous samples, the method should enable thousands to millions of microorganisms to be identified in a cost-effective and timely manner. In addition, because strains of the same species can vary in their ability to colonize roots, antagonize pathogens, or control disease, the method should also be able to differentiate such organisms. Below we discuss some of the advantages and disadvantages of current methods.

DNA nucleotide sequencing. Nucleotide sequence analysis of rRNA genes can be used to obtain detailed phylogenetic depictions of microbial communities. This approach typically involves PCR amplifying rRNA genes from environmental DNA using primers targeting broad taxonomic groups such as bacteria, archaea, or fungi. The resulting amplicons are then cloned, sequenced, and analyzed. The primary limitation of this method is that it usually becomes cost-prohibitive for studies requiring analysis of more than a few samples. Development of less expensive sequencing methods is, however, under way (82), which should eventually lead to a method enabling thorough analysis of microbial community composition in a cost-effective and timely manner.

Array-based methods. Array-based methods, which permit thousands of hybridization events to be examined in parallel, are also being utilized in the field of microbial ecology [for representative studies, see (30, 44, 71)]. Here, labeled rRNA molecules or rRNA genes from environmental samples are

analyzed by hybridization to DNA probes attached to a substrate. These methods allow numerous samples to be examined in a relatively detailed and cost-effective manner. At this time, the most significant factor limiting this approach appears to be probe design. For these methods to work optimally, each probe must hybridize to a specific sequence or group of sequences. However, the development of such a probe set is a challenge, because of the highly conserved nature of rRNA genes, the considerable number of species inhabiting most environments, and the multitude of organisms that have yet to be described. Furthermore, unidentified microorganisms also make it difficult to assess the specificity of probes targeting identified organisms. Large-scale nucleotide sequencing projects that increase the number of rRNA gene sequences should lead to more accurate and comprehensive probe sets, enabling more detailed analyses of microbial community composition.

The OFRG method. An alternative array-based method is termed OFRG (oligonucleotide fingerprinting of ribosomal RNA genes) (10, 98, 99). In the OFRG method, cloned rRNA gene fragments are arrayed on nylon membranes and then subjected to a series of hybridization experiments, each using a single DNA probe, 10 nucleotides in length. For every hybridization experiment, the signal intensities are classified into three discrete values: 0, 1, and N, where 0 and 1, respectively, specify negative and positive hybridization events, and N designates an uncertain assignment. This process creates a hybridization fingerprint for each rRNA gene, which is a vector of values resulting from its hybridizations with all probes. The rRNA genes are identified by clustering their hybridization fingerprints with those of known sequences and by nucleotide sequence analyses of representative rRNA genes within a cluster.

OFRG offers several useful features resulting from the unusual way it classifies gene sequences. Unlike most array-based approaches, which have at least one probe for each target sequence, OFRG uses a small set of probes (~30–50) to coordinately distinguish a much larger set of sequences (for example, all bacterial rRNA genes). The probe sets are designed from training data, which are assemblages of rRNA gene sequences within a defined group. Because the probe sets are typically designed to differentiate all known rRNA genes in a group such as bacteria, the same method is useful for experiments on most any type of sample, from soil to rodents to fruit flies. In addition, because this method analyzes clone libraries, the rRNA gene sequences are readily available for downstream analyses such as sequence-selective qPCR. Finally, and perhaps most important, OFRG can identify rRNA gene sequences that have not been previously described. Even though the OFRG probes are designed from known sequences (the training data), the number of potential fingerprints is exponential with respect to the number of probes, and it significantly exceeds the number of training data sequences. Therefore, fingerprints of these unknown organisms typically fall into taxa that do not cluster with previously identified sequences, and thus can be identified by nucleotide sequence analysis of representative rRNA genes.

At this time, the main limitation of OFRG is its throughput. Although the current method allows 9600 rRNA gene clones to be analyzed per array (7), more throughput is clearly needed. Application of higher throughput techniques to the OFRG method should enhance its capabilities.

Examining all microorganisms. When designing experiments to identify microorganisms involved in specific suppressiveness, another important feature may be to examine all types of microorganisms. Most examinations of the role of microorganisms in suppressiveness have focused on bacteria and fungi. However, it may be just as important to examine archaea, viruses, and other eukaryotic microorganisms. With the exception of viruses,

most of these organisms can be examined using broad-range PCR primers and probes targeting rRNA genes: archaea (5, 28, 40, 59), bacteria (40, 57, 61, 104), eukaryota (27, 32, 40), fungi (8, 11, 38, 53, 60, 85, 89, 112), and oomycetes (4). Additionally, attempts have been made to identify primers and probes targeting all organisms possessing rRNA genes (57, 116).

Analyzing the rRNA internal transcribed spacer region. Given the conserved nature of rRNA sequences, and the range of genotypic and physiological diversity that can exist among organisms possessing similar or identical small-subunit rRNA genes (48, 96), it may also be valuable to examine the more variable intergenic transcribed spacer (ITS) regions located between the rRNA genes (12, 16, 38, 102, 112). This is likely to be especially important for studies of eukaryotic microorganisms, as their rRNA genes typically possess less variation than those from prokaryotes. For example, in the *H. schachtii* studies described above, analysis of the small-subunit rRNA gene identified one phylotype of *D. oviparasitica*. Subsequent analysis of the ITS region has revealed three distinct phylotypes in the 9E field soil (unpublished data), a finding that may be important in fully understanding the nature of the nematode suppressiveness in this soil.

Examining activity levels. Because the number of organisms, or the number of their rRNA genes, may not always be related to their function in the environment, the population-based approach could be modified to examine alternative hypotheses. For example, one hypothesis might be that microorganisms involved in pathogen suppression can be identified by correlating their activity level with levels of the suppressiveness. Here, instead of examining rRNA genes, one could examine levels of rRNA molecules.

Other molecular approaches. Although rRNA gene-based techniques are the most commonly used molecular methods for culture-independent population-based studies, other approaches can also be valuable. Randomly amplified polymorphic DNA (RAPD) analysis has been used to classify 2,4-diacetylphloroglucinate (DAPG)-producing *Pseudomonas* spp. isolated from TAD soils (73). Molecular genotyping methods targeting *phlD* have been used to characterize DAPG-producing bacteria from wheat rhizosphere (64, 65) as well as to track natural populations of these bacteria in corn and soybean fields (63). In addition, subtractive hybridization methods have been used to identify novel markers associated with beneficial microorganisms (50, 62).

Culture-based methods. Culture-based methods remain an essential component of most microbiological investigations. For population-based studies, culture-based methods can be used to identify microorganisms that correlate with specific suppressiveness. For population-based studies utilizing culture-independent methods to analyze microbial community composition, isolating and growing sufficient quantities of the target organisms are critical components of the reintroduction phase. Although many microorganisms have yet to be cultured on laboratory media, recent developments in new types of media and methods have led to considerable advances in this field (18, 26, 47, 49, 51, 76–78, 91, 115).

In addition, to fully utilize the wealth of taxonomic information generated by both culture and culture-independent methods, these data sets need to be connected. For example, in the studies on *H schachtii* described above, the initial BLAST analysis (2) showed that the rRNA genes that consistently and positively correlated with the suppressiveness were related to nematode-destroying fungi. However, only after sequencing rRNA genes from several fungi in our culture collection of nematode-destroying fungi were we able to determine that the suppressive fungus was most closely related to *Dactylella oviparasitica*.

SUMMARY AND CONCLUSIONS

Population-based investigations have contributed substantially to our understanding of specific pathogen suppression in soil. In this chapter, we explored key experimental design principles that guide such investigations. Although these studies face real obstacles, not the least of which is identifying a handful of target organisms among the millions that can inhabit soil, successful outcomes hold considerable potential for plant disease management. Identifying the key suppressive organisms could lead to the development of new strategies to create and maintain suppressiveness and to the ability to more effectively measure disease potential.

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Errata

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