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Mini review

Population dynamics of biocontrol agents and pathogens in soils and rhizospheres

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Abstract

Understanding the dynamics between a pathogen and a biocontrol agent (BCA) in soil or in an infection court such as the rhizosphere is crucial for predicting the success of biological control. This is especially true for biological control using the strategy of reduction of initial inoculum prior to infection. By studying the population density fluxes over time, one can observe how the BCA and pathogen influence each other's population and life cycles, and how the biological and physical environment influence this relationship. Methods for quantifying fungi and bacteria in soil, including classical (dilution plating, baiting, bioassays), immunological (ELISA, IFC, immunomagnetic) and molecular (DNA hybridization, PCR, marker and reporter genes) methods, are discussed and critiqued. Finally, the value of mathematical modeling of population dynamics as a means of providing important information about the tripartite relationship between the pathogen, BCA, and host plant is reviewed.

Abbreviation: BCA – biological control agent; ELISA – enzyme linked immunosorbent assay; GFP – green fluorescent protein; HS – hyperbolic saturation model; ID/DI – inoculum density–disease incidence; IFC – immunofluorescence colony; MPN – most probable number; NE – negative exponential model; PB – probit model; PCR – polymerase chain reaction; Phl – 2,4-diacetylphloroglucinol; RAPD – random amplified polymorphic DNA; SCAR – sequence characterized amplified regions.

Introduction

The discipline of biological control of soilborne plant pathogens can trace its beginnings to Sanford (1926) and Weindling (1932). However, the seminal work that crystallized biocontrol research into a coherent discipline was 'Ecology of Soil-Borne Plant Pathogens: Prelude to Biological Control', based on a meeting in 1963 at Berkeley, California (Baker and Snyder, 1965). In the ensuing years, tremendous strides were made in advancing the science of biological control of plant pathogens, particularly soilborne pathogens. In the past few years, the number of commercially available products has more than doubled, although the total numbers remain small. Every five years, these advances have been chronicled in symposia on soilborne pathogens at the International Congress of Plant Pathology (ICCP) (Hall, 1996; Hornby, 1990; Parker et al., 1985). This review article continues the tradition, arising from a presentation given at the ICPP in Edinburgh, Scotland in August, 1998.

A key to progress in the field of biological control has been understanding the dynamic interactions between the biocontrol agent and the pathogen in the soil and rhizosphere. In the same way that predator–prey relationships have become key in entomological biological

control, knowledge of how the biocontrol agent and pathogen influence each other's populations and life cycles, and how the biological and physical environment influence this relationship is critical. In recent years, there has been a shift toward molecular and biochemical studies and great advances have been made in elucidating the mechanisms of biological control at the genetic and biochemical level. The role of antibiotics, siderophores, competition, and induced resistance in biological control and their genetic regulation have been uncovered using the powerful tools of recombinant DNA, gene sequencing, site-directed mutagenesis, polymerase chain reaction (PCR), and bioinformatics. Despite the opening of these new fronts in genetics and biochemistry, researchers in biocontrol must recognize that pathogens and biocontrol agents (BCAs) exist in populations and that they interact with each other and the biotic environment. There is still merit in pursuing population dynamics studies. Few long-term, comprehensive population studies have been done in the field, partly because of the tremendous sample processing and labor involved, and the move toward more short-term projects as research money has become increasingly limited in many counties.

This review will address the experimental questions that can be answered by studying population dynamics. Current and new methodologies which give a new level of specificity, accuracy, and detection thresholds superior to traditional dilution plating techniques will be described. Once the population dynamics data have been gathered, they can be interpreted using the application of epidemiological models derived from plant pathology. These models allow one to look at the interaction of the BCA and pathogen over a wide range of population densities and to consider plant host factors.

Experimental questions about the pathogen

Studies of population dynamics can answer basic questions of how many, when, and where, or in more scientific terms, they can describe the inoculum density and the temporal and spatial aspects of inoculum.

The relationship between inoculum density of the pathogen and disease incidence is key to understanding and predicting the outcome of disease. Some of the principles of this relationship, including the mathematics and graphical representation of the inoculum density–disease incidence (ID/DI) curve were outlined by Baker (1978) (Figure 1). The idealized ID/DI curve can be represented by a sigmoidal curve: in general,

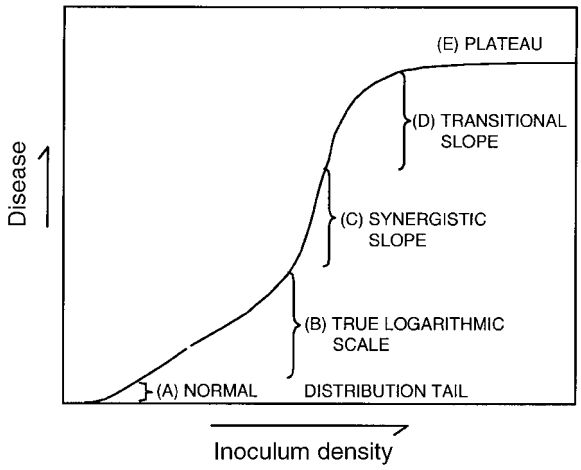

Figure 1. Inoculum density–disease incidence curve.

the more inoculum, the more disease. But as the inoculum increases, the slope of the curve decreases to an asymptote as the efficiency of the inoculum decreases. This decrease in efficiency is due to multiple infections, propagules competing for infection sites, and a reduction in available host tissue. These curves provide useful information on the effects of the environment or of control strategies such as biological or chemical control. These curves are often transformed and linearized with Gregory's multiple infection transformation or probit transformations (Baker, 1978). The information can be used to predict the potential for disease in the field before planting, so growers can consider various management options such as biological control, resistant varieties, crop rotation, and chemical control. This allows disease thresholds to be established. How much inoculum is needed before the disease is noticeable? What is the action threshold, when the treatment must be applied? What is the economic threshold, where the cost of treatment equals the potential crop loss? Such thresholds have been established for many foliar diseases, but for only a few soilborne ones. Nicot and Rouse (1987) established that ≥ 6 propagules/g of soil of *Verticillium dahliae* would cause potato early dying, although when *Meloidogyne hapla* was present, populations below this threshold caused disease (MacGuidwin and Rouse, 1990).

How long does inoculum survive in the presence or absence of the host? Benson (1994) discussed idealized survival curves of soilborne inoculum with an initial survival phase, logarithmic death phase and a residual survival phase. How does the pathogen spread

in time and space? Very few spatio-temporal studies have been done with soilborne pathogens, where both time and space are examined. Unlike foliar pathogens, soilborne pathogens spread slowly over time, so one has to observe many years to see the spatial spread of the disease or work with microcosms. The typical representation of spread over time has been the disease progress curve, in which the amount of disease is plotted as a function of time. This usually produces a monomolecular curve for monocyclic 'simple interest' root diseases or a sigmoidal curve when secondary infection occurs. These curves have been modeled with pathogen and host factors, including the dynamics of inoculum production, the rate of contact between inoculum and roots, root density and root growth (Gilligan, 1994). In more recent work, the effect of the initial inoculum density on the temporal spread of the take-all fungus, *Gaeumannomyces graminis* var. *tritici* was described with experimentation and mathematical modeling (Bailey and Gilligan, 1999). High inoculum densities produced a monotonic disease progress curve, but lower inoculum densities produced a curve that was initially monotonic with a plateau, and then increased sigmoidally to an asymptote. This dual phase curve was attributed to primary and secondary infections, inoculum decay, and growth of the host plant.

Studies of spatial patterns of soilborne pathogens and disease have been facilitated by spatial autocorrelation and geostatistical techniques. For example, Xiao et al. (1997) examined the patterns of microsclerotia of *Verticillium* in the soil and the pattern of wilted cauliflower in the field.

Combining spatial and temporal aspects of disease dynamics is complex. Several scientists studied these aspects for foliar diseases (Gottwald et al., 1992; Reynolds et al., 1988), but few researchers have attempted studying these interactions with soilborne pathogens, as did Mihail (1989) and Campbell and van der Gaag (1993) with *Macrophomina phaseolina*. Gilligan (1995) proposed the use of reaction–diffusion models to link the spatial and temporal dynamics of disease.

Information about the spatial distribution of soilborne pathogens is crucial for the development of sampling techniques that balance statistical reliablity with cost. The spatial pattern of most root diseases is usually aggregated (Campbell and Noe, 1985) and a systematic pattern of sampling provides a better estimate than random sampling methods (Campbell and Neher, 1994). The number of samples required for a representative sample can be estimated from the standard error or coefficient of variability, from probability statements, or from balancing the variance components with the cost components to arrive at an optimal number (Campbell and Madden, 1990). However, few papers have rigorously tested sampling techniques for soilborne pathogens, especially when detection is based on molecular methods.

Experimental questions about the BCA

Population dynamics studies provide crucial facts about the BCA. One is the level of survival of the BCA in the formulation or substrate prior to use. Our laboratory has been trying to improve the survival of *Pseudomonas aureofaciens* 63-28 in peat planting mixes. This bacterium produces a novel class of antibiotics (Gamard et al., 1997), and is an effective BCA against *Pythium* and *Rhizoctonia* (Paulitz, unpublished, Seresinhe et al., 1997). An ideal product would have the bacterium formulated in the growing mix. However, population experiments showed that *P. aureofaciens* did not survive at a critical threshold of 10^6 cells/g mix for more than 2–3 months in peat at 45% moisture, the moisture level at which it is sold. Moisture is the major environmental limitation. The effect of different adjuvants, i.e. osmotic stabilizers, protectants and cultural conditions was studied. Freeze drying or formulations in vermiculite gave survival rates of six months to one year, if stored dry. However, if added to the peat growing mix at 45% moisture, the populations declined in 2–4 months.

Since most BCAs are applied in an inundative strategy, it is important to maintain high populations long enough to have an effect on the pathogen. Most BCAs have a threshold level needed for biocontrol, and the initial population of the BCA can affect the level of biocontrol (Bull et al., 1991). BCA populations are affected by environmental conditions and have an optimum environmental window for the best efficacy. The optimal conditions can be defined with population studies. A further question now emerging that may be more important than actual numbers is the activity of the BCA: is it in a metabolically active state producing the antibiotic or siderophore needed to antagonize the pathogen? Some methods for studying both BCA activity and populations will be described in this paper.

How do the pathogen and BCA interact at the population level? If mycoparasitism was operating, one would expect a decline in the pathogen population concomitant with an increase in the mycoparasite

population. If competition was important, one would expect a decline in the carrying capacity of the system with both organisms, compared to either alone. Replacement series experiments, where one population is held constant and the other is varied, is one way of looking at competition. Important questions include: (1) What effect does the population density of the BCA have on the pathogen, i.e. how does the pathogen respond to different levels of the BCA? (2) What effect does the population density of the pathogen have on the BCA? This is important for mycoparasites and other BCAs that depend on the pathogen for their survival. (3) What is the efficiency of the BCA in controlling the pathogen, i.e. how many BCA propagules does it take to eliminate one propagule of the pathogen? (4) What is the maximum level of pathogen pressure under which the BCA can give adequate control? Many of these questions will be addressed in the last section on modeling of pathogen/BCA interactions.

Methods for quantifying fungi

Each method has its advantages and disadvantages. Which one is best will depend on each situation. It is a balance of cost, accuracy, precision and reproducibility.

Direct quantification. Some fungi form large propagules that can be directly quantified. For example, sclerotia of *Sclerotinia sclerotiorum* or *Sclerotium rolfsii* can be wet sieved from soil. Recovered sclerotia can also be tested for viability.

Dilution plating. The most widely used method for quantifying fungi is the dilution plate/selective medium method, which is based on suspending soil particles in water and spreading on a Petri plate. Excellent selective media exist for many of the most widely studied soilborne pathogens, including *Pythium*, *Phytophthora*, *Fusarium* and *Rhizoctonia* (Dhingra and Sinclair, 1995; Singleton et al., 1992). Most of these media are really semi-selective, by using selective inhibition of unwanted non-target organisms. For example, antibiotics such as pimaricin inhibit non-Oomycetes, while streptomycin, penicillin and chloramphenicol inhibit bacteria. Fungicides such as benomyl inhibit Ascomycetes and Deuteromyces, but not Oomycetes. Another strategy is to use a substrate that only the target organism can utilize. For example, cellulolytic fungi can be assessed on media containing cellulose. Selective media can also be developed that result in a unique colony morphology of the target organism, which can be easily recognized. Finally, some media use an environmental extreme that the target organism is tolerant of and non-target organisms are sensitive to, for example, high salinity or high temperature.

Dilution plating has several inherent disadvantages. It cannot distinguish closely-related fungi, for example, pathogenic and non-pathogenic isolates of *Fusarium oxysporum*. Some fungi are slow-growing and cannot compete with faster growing fungi. Finally, the method can overestimate fungi that sporulate heavily. This method estimates colony forming units (cfu), and this may not be correlated with actual biomass. Nevertheless, many samples can be processed, using a minimum investment in equipment. Automation, including spiral plate devices, is making this method even less labor intensive. Spiral plate devices can do many dilutions on a single plate (Schober and van Vuurde, 1997), and automatic plate pouring devices reduce the time needed to prepare the media. Plating techniques can be as sensitive as 1 cfu/ml, more sensitive than serological techniques, but only with a low level of background organisms.

Baiting. This method involves the use of living or non-living plant substrates, e.g. leaf pieces, cotyledons, seedlings or fruit, added to a soil sample. It is used for zoosporic pathogens such as *Pythium* and *Phytophthora* that are difficult to isolate with selective media. The baiting substrate is put into flooded soil and zoospores are attracted to it. The substrate can then be placed on selective media where the fungus will grow readily. The advantage is that the bait can detect low inoculum levels. For example, in hydroponic studies with *Pythium aphanidermatum*, millet seeds were used to bait the fungus out of water samples and quantify the inoculum of the pathogen (Rankin and Paulitz, 1994). This method can be made semi-quantitative by developing standard curves with known zoospore concentrations (Rankin, 1992).

Bioassays. Obligate soilborne pathogens, such as *Plasmodiophora brassica* and obligate symbionts, such as arbuscular mycorrhizal fungi, can only be quantified with bioassays. This method can also distinguish between pathogenic and non-pathogenic races and formae speciales of fungi. The numbers can be quantified with the use of most probable number (MPN) statistics,

based on the presence or absence of the infection in bioassay plants in serial dilutions of the soil. The advantages are its sensitivity and ability to determine the inoculum potential or infectivity present in the sample, an important factor in predicting the resulting disease. It can be used to measure the soil receptivity to a pathogen (i.e. conduciveness or suppressiveness) (Oyarzun et al., 1997). However, it is costly and labor intensive, since plants must be grown under controlled conditions.

Immunological techniques. In contrast to dilution plating, immunological techniques are insensitive to microbial backgrounds and non-target organisms. Immunological techniques can also be species-specific, eliminating the error of distinguishing microbes closely related to the target. However, the cost of developing an antibody is high and an extensive collection of fungi has to be tested to be sure that the antibody is specific. Unlike viruses and bacteria, relatively few immunological tests have been developed for fungi. Monoclonal antibodies are used in commercial ELISA detection kits for soilborne plant pathogenic fungi such as *Pythium*, *Phytophthora*, *Sclerotinia* and *Rhizoctonia* (Miller, 1996; Miller et al., 1997; Timmer et al., 1993, Ali-Shtayeh et al., 1991). Monoclonal antibodies have been combined with immuno-magnetic bead assays (Thornton, 1996) and baiting (Thornton et al., 1999) to increase the sensitivity and recovery of *Rhizoctonia solani* from soil. Monoclonals have the advantage of being more specific and have less cross-reactivity than polyclonal antibodies. The use of monoclonal antibodies to detect soilborne fungi has been reviewed by Dewey et al. (1997).

Marked strains. One approach to ecological studies of fungi is to transform them to a phenotype enabling them to be selectively isolated or visualized. An example is the β-glucuronidase (GUS) reporter gene from *Escherichia coli*. This gene can be transformed into fungi and the enzyme produced can be quantified colorimetrically by the addition of the appropriate substrate, such as X-Gluc. Only metabolically active cells are detected. Eparvier and Alabouvette (1994) used GUS-marked strains of pathogenic and non-pathogenic *F. oxysporum* to study their interaction in flax roots. Hygromycin resistance, coded by hygromycin B phosphotransferase, can also be used. Lo et al. (1998) used both GUS and hygromycin resistance in a strain of *Trichoderma harzianum* to study its growth in the rhizosphere and phyllosphere. Hygromycin resistance was used to monitor the population density of *Trichoderma* and visualization of the blue-stained hyphae was used to study the microscopic interactions between *T. harzianum* and the pathogen *R. solani*. Green and Jensen (1995) used GUS-marked strains to study *T. harzianum* in the rhizosphere of cucumber. Bao (1999) optimized the method to study the biomass of non-pathogenic strains of *F. oxysporum* in the roots of tomato. The fungal biomass detected by the GUS method was 8–80 times greater than the numbers estimated from dilution plating.

DNA based techniques

DNA hybridization assays. These methods use DNA probes based on complementary DNA sequences present in the target pathogen. The probe can be labeled with radioactive ^{32}P or non-radioactive probes such as biotin or digoxygenin, and hybridized with the unknown sample on membranes as a dot blot or as a Southern blot. This methodology was used to detect *Phytophthora parasitica* (Goodwin et al., 1989) and *P. citrophthora* (Goodwin et al., 1990). However, these methodologies are not suitable for quantification and many require extraction of the DNA from the cultured organism.

PCR methods. The PCR (Henson and French, 1993), is a method of producing probes with less cost and time. It gets around the problem of limited amounts of DNA because the method amplifies the specific sequence of interest. For example, Lévesque et al. (1998) developed a reverse dot-blot hybridization to detect different species of *Pythium* on one blot. PCR was used to amplify the ITS regions that were species-specific. These internal transcribed spacers (ITS) are found in rDNA and are used for taxonomic studies, since they are repetitive and contain both highly conserved and highly variable areas. Another state-of-the-art technology called microarray involves applying hundreds or thousands of different oligonucleotides to a chip and may represent the future for this type of molecular diagnosis, but this technique is not quantitative in its present form (Lemieux et al., 1998; Kurian et al., 1999; Lipshutz et al., 1999).

Another widely used PCR method is random amplified polymorphic DNA (RAPD). It uses small 9- to 10-base oligonucleotides that randomly amplify unknown sequences of the target DNA. It has the

advantage that knowledge of the target is not needed. However a pure culture of the fungus is required. The amplified DNA can be sequenced to produce specific primers called SCARs (sequence characterized amplified regions), increasing the reproducibility of the method. PCR techniques have been used to detect soilborne fungi such as *Phytophthora* (Lee et al., 1993), *Fusarium graminearum* (Schilling et al., 1996), *Verticillium* (Volossiouk et al., 1995), *R. solani* (Bounou et al., 1999) and *Monosporascus* (Lovic et al., 1996).

What are the advantages of PCR methods for population studies? They are very specific, species or even strain-specific. The methods are extremely sensitive and can detect very small populations. However, there are several obstacles to using PCR for population studies. Specialized equipment is required, including a thermal cycler and electorphoresis equipment and there are significant labor costs. Another major problem lies in extracting DNA from soil or plant samples. Soil contains humic substances that can interfere with the PCR process, but the DNA can be cleaned up with cesium chloride gradients and other methods. The PCR products have to be run on electrophoresis gels to be identified. All this limits the numbers of samples that can be run in a day. PCR can also amplify dead or non-viable organisms and non-target DNA, if certain precautions are not taken. However, the rate of breakdown of DNA from dead fungi may be fairly high (Herdina et al., 1999), so amplification of DNA from dead cells may be a minor problem. A major limitation in population studies has been the problem of DNA quantification with PCR. Because PCR is an exponential process, estimating the initial amount of DNA in the sample from the amplified amount is difficult. Any small variation in the amplifying efficiency from one run to the next can result in large differences in PCR product yield. One way of quantifying DNA is to dilute the sample until no PCR product is amplified, and then using MPN statistics. However, the large number of dilutions and replications makes this labor intensive. The other method is competitive PCR, where an internal standard (competitor) with a known DNA sequence and mass is added to the original sample. The competitor and target are amplified together, using the same primers, and the ratio of the final mass of the competitor is compared to its starting mass, enabling the user to estimate the amount of original target from the final amount. This method assumes that the efficiency of amplification of the primer and competitor are the same, which is only true within a narrow range of ratios. Competitive PCR has been used to quantify the DNA of *Leptosphaeria maculans* in oilseed rape during disease development (Mahuku et al., 1995), the amount of *Verticillium* in plants (Hu et al., 1993), or the amount of the bacterium *Clavibacter michiganensis* subsp. *sepedonicus* in potatoes (Hu et al., 1995).

Another limitation is the need to run the PCR products on electrophoresis gels. A technique called PCR-ELISA eliminates this problem. First marketed by Boehringer Mannheim, this involves labeling the PCR primers with digoxygenin (DIG) or biotin. The sensitivity of this method is 10–100 times greater than electrophoresis gels. The system can be made quantitative by running dilutions of controls with known amounts of target DNA. This method is very popular in medical and veterinary microbiology, but is only recently gaining use in phytopathology. Poggi Pollini et al. (1997) used this method to detect phytoplasmas in trees and shrubs and the technique is being developed to detect *R. solani* AG-3 from soil (Leblanc et al., 1999) and *Clavibacter michiganensis* subsp. *sepedonicus* in potato cores (Mills and Russell, 1999).

Another system called TaqMan® (Perkin Elmer, Inc) also eliminates the need for gels. This uses another probe that hybridizes to a target on the PCR product. The probe contains a reporter dye and a quencher dye. When the product is amplified, the 5′ nuclease activity of the DNA polymerase cleaves the fluorescent probe away from the quencher, so the fluorescence of the reporter is increased. The more the target is amplified, the more dye is released. The dye can be quantified in a spectrophotometer built into the PCR machine. This technique has been used to detect a potato virus (Schoen et al., 1996).

A variation of this technique, called real-time PCR or kinetic PCR, makes it even more quantitative (Higuchi et al., 1993). This method has not yet been applied to quantification of plant pathogens, probably due to the high cost of the equipment at the present time. However, the method has been used to select more sensitive primers for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* using BIO-PCR and TaqMan (Schaad et al., 1999). The increase in DNA product is fluorogenically measured in each cycle. From this curve, the threshold cycle can be determined, when the fluorescence generated by the cleavage of the probes passes a threshold level. This threshold cycle can be correlated to the copy number using a standard curve. Thus the

estimation is based on the beginning of the cycle, not the end.

Methodologies for quantifying bacteria

Dilution plating and antibiotic resistance

As with fungi, dilution plating has been the mainstay of bacterial population studies. The most common methods use antibiotic-resistant strains of bacteria. These strains can either be selected as spontaneous chromosomal mutants or engineered with the antibiotic resistance gene. Rifampicin and naldixic acid are commonly used, because there is little background resistance to these antibiotics in soil bacteria. However, antibiotic resistance can have pleiotrophic effects on the growth and competitiveness of the marked strains. A study by Mahaffee et al. (1997) compared different methods of assaying a biocontrol strain of *Pseudomonas fluorescens* in a cucumber field. The three methods used were plating of strains marked with rifampicin resistance on rif medium, visualization of strains genetically engineered with the bioluminescent lux gene, and the quantification of strains with the immunofluorescence colony (IFC) staining technique. They consistently found higher populations with the IFC method, suggesting that the genetically altered bacteria may have reduced fitness and thus the population may have been underestimated.

Immunological techniques

Immunological techniques such as ELISA have been more widely used with bacteria than fungi, because of the greater specificity of bacterial antigens. One advantage of these techniques is that the wild type strain is not genetically altered. A more sensitive technique called IFC staining, combines isolation with serological colony identification (van Vuurde, 1990). With the IFC staining technique, the antibody is conjugated with fluorescein isothiocyanate. The bacteria are plated out and the target colonies are distinguished from non-target bacteria, using incident blue light and the appropriate filters. This method also allows for *in situ* visualization of bacteria in the plant. It can be almost as sensitive as PCR, detecting 10^2 cells/ml (Schober and van Vuurde, 1997). Bacteria can be further tested by PCR or fatty acid analysis to confirm their identity. Immunomagnetic attraction and fishing are immunocapture techniques for enriching the target bacterium, and increasing the chances of detection in mixtures with non-target organisms (Jones and van Vuurde, 1996). The suspension is mixed with paramagnetic iron oxide particles coated with a specific antibody, to which the bacteria attach. The particles are removed from the solution with a supermagnet and streaked on agar to dislodge the bacteria. This technique can be combined with other methods such as PCR to increase the detection sensitivity of the method. *Erwinia caratovora* subsp. *atroseptica* was detected in potato peel extracts at 2×10^3 cells/ml, when the immunomagnetic method was combined with PCR (van der Wolf et al., 1996).

Molecular techniques

RNA-directed probes. RNA-directed probes against specific sequences of rRNA have been used to detect and quantify bacteria (Hahn et al., 1994). With this *in situ* labeling technique, the oligonucleotide probe is labeled with a fluorochrome and penetrates into the cell. This method has the advantage of being able to detect non-culturable but active bacteria. It is widely used in soil microbiology and microbial ecology to analyze community structures, but only a few studies have looked at plant-associated bacteria (Assmus et al., 1995).

Marker and reporter genes. Marker genes can be introduced into bacteria to give the organisms a unique physiological property. The assumption is that the background organisms do not possess this activity and that the marker gene does not alter the fitness of the bacterium. These markers can also be used as reporter genes, when they lack their own promoter, but are placed downstream from an exogenous promoter, so they can 'report' on the activity of that promoter. These promoters can regulate important biocontrol functions such as siderophore or antibiotic production. Thus, reporter genes can give an insight into the environmental conditions that may be conducive for biocontrol activity. Reporter genes can also give an indication of the activity of a biocontrol agent, which may be as important as the population density.

LacZ and gusA. *LacZ* encodes for the β-galactosidases, which cleave lactose into glucose and galactose, while *gusA* encodes for β-glucuronidase. β-galactosidase activity can be detected by a colorimetric reaction

with the substrate X-Gal, producing a blue color. This method was used to track genetically engineered bacteria released into the environment (Kluepfel et al., 1991). Soil bacteria do not have β-galactosidase activity, reducing the problem of background interference.

GFP – green fluorescent protein. This protein, produced by a jellyfish, absorbs violet light and fluoresces green. It is amenable to direct *in situ* observation with confocal laser microscopy or epifluorescence microscopy. GFP has been used as a marker to quantify *Aureobasidium pullulans* on leaf surfaces (van Wymelenberg et al., 1997) and to quantify and follow *Cochliobolus heterostrophus* in maize leaves (Maor et al., 1998).

Lux. This is a series of bioluminescence genes from the marine bacterium *Vibrio fischeri*. The genes code for the production of luciferin and luciferinase. If only the genes for luciferinase are put in the bacterium, exogenous aldehyde substrate must be provided for light emission, which can be quantified with a scintillation counter, luminometer, X-ray film or charge-coupled device (CCD) cameras which can detect low levels of light. This method has been used to study the release of genetically-engineered bacteria in the field (Shaw et al., 1992), root colonization by *Pseudomonas* spp. (de Weger et al., 1991), soil microbes and the activity of *P. fluorescens* in the soil (Meikle et al., 1992) and to visualize bacteria *in planta* (Dane and Shaw, 1993). It can have a sensitivity limit of 1.5×10^4 cfu/leaf (Shaw et al., 1992), but single cells can be detected in soil samples (Silcock et al., 1992). Its advantage is that the spatial and temporal dynamics of the bacteria or expression of genes can be visualized. This gene may make the target bacterium less fit, since the reaction requires reducing power and energy, and the bacteria have to be actively growing to emit light. Light can also be quenched by particles in soil, making visualization difficult.

inaZ. This reporter gene is based on a gene that produces an ice nucleation protein (Loper and Lindow, 1997). It can be quantified by a simple freezing assay. The number of cells is correlated with the number of ice nuclei in the sample, which is related to freezing temperature. As few as 10 cells can be detected in a sample. The expression of ice nucleation does not require high metabolic activity and can be expressed in the exponential or stationary phase of the bacterium. An *inaZ* reporter gene system was used to look at the expression of pyoluteorin biosynthesis genes over time by *P. fluorescens* on cucumber and cotton seed planted in soil (Kraus and Loper, 1995). The bacterium established a larger population in the spermosphere of cotton and the expression of the pyoluteorin genes was delayed on cucumber, suggesting a nutritional effect on antibiotic production.

PCR

PCR can be used to detect and quantify bacteria (see section on PCR-fungi) and also to target specific genes for biocontrol functions. For example, Raaijmakers and Weller (1998) used PCR primers that targeted the gene for 2,4-diacetylphloroglucinol (Phl). They found that Phl-producing *Pseudomonas* spp. were associated with suppression of take-all, caused by *Gaeumannomyces graminis* var. *tritici*.

Modeling biological control

In the previous sections, I reviewed some of the current methodologies that are available to measure the population dynamics of pathogens and BCAs over time and space. How can data from these techniques be used to examine the interaction between the two microbes? One way is to apply epidemiological principles derived from plant pathology. Johnson (1994) recognized that the same principles that could be applied to the interactions between pathogens and their host plants could be applied to BCA and their host, the plant pathogen. He looked at the ID/DI curves, which could be modeled by a negative exponential model. This model is linearized by Gregory's multiple infection correction $\ln 1/(1-Y)$, along with a function to bring the asymptote lower than 100%. In his final model, $y = 1 - \exp\{-kx[(1 - A) + A \times \exp(-cz)]\}$, where A is an asymptote variable (the maximum proportion of the pathogen population that can be rendered ineffective by a BCA), k and c are two efficiency variables for the pathogen and BCA, respectively; and x is the inoculum density of the pathogen and z is the inoculum density of the BCA. Johnson applied this model to the data of Mandeel and Baker (1991) (Figure 2), who examined the effect of inoculum density of *F. oxysporum* f. sp. *cucumerinum* on the development of wilt of cucumber in soils amended with different inoculum densities of two isolates of non-pathogenic *F. oxysporum* (C5 and C14). Using a Gompertz transformation (Berger, 1981) to estimate

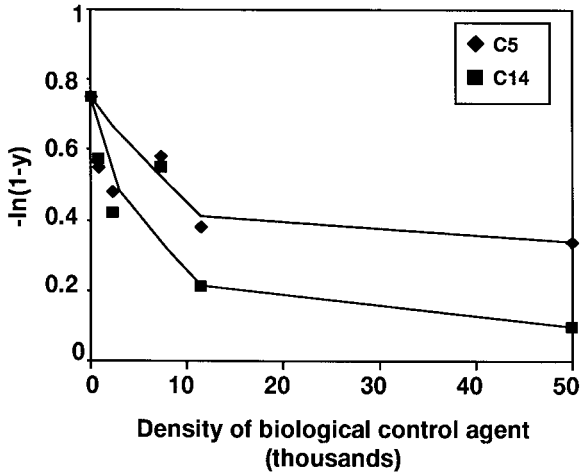

Figure 2. Effect of non-pathogenic *F. oxysporum* isolates C5 and C14 on the proportion of cucumber plants diseased, y, with Fusarium wilt 55 days after planting. Density of the pathogen, *F. o. cucumerinum*, x was 2000 cfu/g. Data points were obtained from Mandeel and Baker (1991). Curves were fit to the data based on the equation $y = 1 - \exp\{-kx[(1 - A) + A \times \exp(-cz)]\}$, where z is the density of isolate C5 or C14, k and c are efficiency constants with values of 0.00037 and 0.00015, respectively, and A is an asympote parameter with values of 0.582 for C5 and 0.893 for C14. Graph taken from Johnson (1994) with permission of author and journal.

the efficiency constants and asymptote constant, isolate C14 had the same efficiency as isolate C5 in reducing the effects of the pathogen inoculum, but isolate C14 had a lower A value, indicating that it rendered a higher proportion of the pathogen inoculum ineffective. This model was recently validated using crown gall (*Agrobacterium tumefaciens*) on tomato and cherry, controlled by *Agrobacterium radiobacter* strain K84 (Johnson and DiLeone, 1999). To examine the role of the antibiotic agrocin in biological control, they used strains of *A. tumefaciens* sensitive and resistant to the antibiotic, as well as antibiotic producing and non-producing strains of *A. radiobacter* K84. The only parameter that differed significantly among the treatments was the asymptote parameter A, indicating that with a resistant strain of *A. tumefaciens* or an agrocin 84-deficient strain of *A. radiobacter*, the maximum level of control was reduced.

Raaijmakers et al. (1995) examined the dose-response relationships in the suppression of Fusarium wilt of radish by *Pseudomonas putida* and *P. fluorescens*. They looked at the absolute reduction in disease at different levels of the pathogen, and found a quadratic relationship, with little reduction at high and low levels of disease but the maximum reductions at mid-range levels of disease. But when looking at the relationship between relative disease reduction and disease levels in the non-treated, inoculated control, the relationship was linear, with the most disease reduction occurring at low disease levels. The results express mathematically what many researchers intuitively assumed: that at high disease levels BCAs are less effective. Studies of the relationship between disease reduction and the population density of the biocontrol agents revealed a non-linear, asymptotic relationship, with a threshold of approximately 10^5 cfu/g of root required for biocontrol. Increasing the population density of the BCA to higher levels did not result in a significant improvement in disease control. Montesinos and Bonaterra (1996) extended the idea further by testing not only the negative exponential (NE) model, but also a probit (PB) and hyperbolic saturation (HS) model (Schneider, 1984). This is the same type of first order kinetics seen with enzyme reactions. At higher inoculum densities, the pathogen targets of the BCA become saturated. The HS model gives an ED_{50}value for both the pathogen and BCA (K_x and K_z), the asymptotic level of disease without the BCA (Y_{max}), the maximum proportion of the pathogen inactivated by the BCA, and the ED_{50} ratio of the BCA/pathogen. This last ratio is similar to the mycoparasite efficiencies proposed by Adams (1990). Put simply, how many BCA propagules does it take to inactive one pathogen propagule? Efficiencies ranged from 1.6 for the *Sporidesmium sclerotivorum*/*Sclerotinia minor* system to 5×10^4 for *Trichoderma* spp./*R. solani*. Montesinos and Bonaterra (1996) tested their models with the data on the biocontrol of *Stemphylium vesicarium* on pear leaves with *Pseudomonas* and *Erwinia* spp. and data of Mandeel and Baker (1991). All three models fit the data well, and the HB and PB model showed an ED_{50} of the nonpathogenic *F. oxysporum* to be 2×10^3 cfu/ml, while for the bacteria in the *Stemphylium* system, 6×10^6 cfu/ml were required. Smith et al. (1997) built on these ideas by incorporating a host component and tried to separate the effect of the host plant, both in terms of its inherent disease resistance and its ability to support the BCA. They examined the NE, HS model and a logistic model, and compared their goodness of fit. They worked with eight plant species and three *Pythium* spp. at different inoculum densities. To narrow it down to a cultivar effect, they looked at six different inbred lines of tomato inoculated with one species of *Pythium* over a range of

inoculum densities and different doses of the BCA, *Bacillus cereus* UW85. The response of the cultivars to the pathogens (ie. resistance response) was different than their responses to the BCA. In other words, there appears to be an inherent difference in the ability of a plant to support biological control, or host hospitality, a genetic trait that may be bred and selected for.

The interactions of BCAs and pathogens over time have been modeled by C. Gilligan's group. In many cases in the literature, the populations of pathogens or BCAs show over time a non-monotonic curve, initially increasing with time, and then subsequently declining to an asymptotic equilibrium. A non-linear, critically damped model was developed and applied it to data sets from the literature (Gilligan, 1990). These included the pre-emergence damping-off caused by *R. solani* in successive crops of lettuce, the population dynamics of *Trichoderma viride* in plant growth media, the population of *Pythium ultimum* in the presence and absence of *Laetisaria arvalis*, and the colonization of bean fragments by *P. ultimum* in the presence and absence of the mycoparasite *Pythium nunn*. None of the biocontrol treatments affected the rate parameters, but they did influence the locational and asymptote parameters. In other words, the rate of decline of the pathogen was not affected, but the final asymptotic equilibrium level was reduced and the time at which maximum colonization or populations were attained was shifted.

How do BCAs and pathogens affect the probability of pathogen infection at different distances from the host in the soil? Bailey and Gilligan (1997) demonstrated that *T. viride* changed the pathozone profile of *R. solani* over time. The pathozone is the volume of soil around an underground plant organ in which a propagule must lie if it is to have a chance of infecting the plant (Gilligan and Bailey, 1997). The probability of infection varies with the distance between the propagule and the plant. This change in probability over distance is the pathozone profile. It was measured by placing 1-mm disks of *R. solani* from PDA at various distances from germinating radish seeds in sand. The pathozone profiles were non-monotonic with the maximum chance for infection close to the root (2-mm away) but not touching the host. The probability of infection decreased asymptotically to zero as the distance from the host increased. The maximum extent of pathozone influence increased over time in a monomolecular function reaching an asymptote. *T. viride* reduced the maximum extent of the pathozone from 22.6 to 13.8 mm. The change in pathozone profiles over time was also used to predict the disease progress curves for the disease. These curves showed a maximum incidence of disease of 42% in the absence of *Trichoderma*, but 13% in the presence of *Trichoderma*.

Conclusions

An examination and investigation of the population dynamics of pathogens and BCAs can provide critical information that can be applied to the management of pathogens, whether by biological or other methods. Methodologies are evolving, each with its own set of advantages and drawbacks. Molecular methods have greater sensitivity and specificity than earlier techniques, but have not been widely applied in extensive population studies. Application of epidemiological and mathematical models to data should help decipher the complexities of these interactions and reveal fundamental ecological and biological principles that underlie the thrust and parry of microbial warfare.

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