THE SCREENING OF MICROORGANISMS ANTAGONISTIC TO PHYTOPATHOGENS

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ABSTRACT - There are two general options for screening potential biocontrol agents to a plant pathogen: First, microbes can be tested for antagonism under controlled conditions (in the laboratory and/or in a growth chamber or greenhouse), followed by assay of the promising isolates under uncontrolled conditions in the field for final evaluation. This approach offers convenience and efficiency, standardization and reproducibility of test conditions, and the opportunity to directly examine the antagonist/pathogen interaction. However, tests under controlled conditions may fail to detect antagonism or may be poor predictors of biocontrol activity under field conditions. Second, microbes can be tested for activity under uncontrolled conditions, directly in the field, without preliminary assay. This approach offers rigorous, realistic appraisal of biocontrol potential. However, it poses several logistical constraints even if micro-plots are used, it may not be representative of activity at other times or locations, and it offers no clue as to the underlying mechanism. Both approaches have advantages and disadvantages and choosing either involves compromises. Any screen may be case-specific and may be valid only for those conditions imposed by the protocol. Which strategy will ultimately provide for the optimal evaluation, offering the highest predictability per unit cost invested in time and material, remains to be determined.

Index terms: biological control, plant pathogens, antagonists, antagonism, disease control.

SELEÇÃO DE MICRORGANISMOS ANTAGÔNICOS AOS FITOPATÓGENOS

RESUMO – Em geral, existem duas opções para a seleção de agentes de controle biológico de fitopatógenos. Na primeira opção, os organismos podem ser testados como antagônicos em condições controladas (no laboratório e/ou câmara de crescimento ou em casa de vegetação). A seguir, os isolados mais promissores são testados em condições de campo para uma avaliação final. Este método oferece conveniência, eficiência, padronização e reprodutibilidade das condições de testes, e a oportunidade de examinar diretamente as interações patógeno/antagonista. No entanto, testes sob condições controladas podem falhar na detecção de antagonistas, ou podem ser fracos indicadores da atividade do controle biológico em condições de campo. Na segunda opção, a atividade dos microrganismos pode ser testada em condições não controladas, diretamente no campo, sem testes preliminares. Este método oferece avaliação rigorosa e realista do potencial do controle biológico. Entretanto, ele apresenta várias dificuldades logísticas, mesmo quando microparcelas são usadas, não deve ser representativo de atividade em outros locais e épocas, e não oferece indicações sobre os mecanismos fundamentais. Ambos os métodos têm vantagens e desvantagens, e a escolha de cada um envolve compromissos. Qualquer seleção deve ser específica para cada caso, e deve ser válida somente para aquelas condições impostas pelo protocolo. Que estratégia proverá, em última análise, para uma ótima avaliação, oferecendo as melhores condições de previsibilidade por unidade de custo investido em tempo e material, ainda está por ser determinado.

Termos para indexação: controle biológico, doenças de plantas, antagonistas, antagonismo, controle de doenças.

INTRODUCTION

There is no aspect of microbial biocontrol more important, and probably none more controversial, than the screening of candidate microbes. Basically, there are two issues: 1) Where does one obtain such microorganisms; and 2) How does one efficiently assay the pool of candidates in a manner that reliably

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predicts performance in the field under prevailing cultural practices. Efficiency here refers to a protocol that minimizes the number of false positives (presumptively active candidates which fail in the field) and false negatives (effective field agents mistakenly discarded based on misleading preliminary results) per unit cost (time, resources) invested in the screen.

This paper is organized as follows. I begin by considering possible sources of candidate microbes. The different components of a screen are then compared briefly. A screening sequence that could be implemented efficiently is then proposed. I conclude by providing an overview of the options. Thus, the paper considers only how to find biocontrol agents, not how to improve or modify candidates once they are found. Most of my examples are drawn from phyllosphere research which is our main area of interest. Clearly, however, the principle of screening has many common properties, regardless whether screens are designed to sort out biocontrol agents (broadly interpreted to mean microbes active against any plant pathogen, insect, or weed); growth promoting microbes (e.g., mycorrhizal strains); resistant plants; or even prospective versus unlikely sites for drilling or mining ventures.

SOURCES OF MICROORGANISMS FOR BIOLOGICAL CONTROL

No microorganism should be overlooked in the search for prospective biocontrol agents. The premise for this is that we still have little sound knowledge of the microbial features that confer successful biocontrol in nature. Moreover, even if a microbe can not antagonize effectively under field conditions, it still could be a source of genes to convert a non-antagonist to a useful biocontrol agent (hence, as discussed later, one reason to retain a controlled environment assay as part of the screening protocol).

While the search for agents should ideally be unconstrained, resources are limited in practice and there are ways to increase the probability of success. Screens can be focused in two ways, based either on the presumed colonizing potential of the microbe (Table 1) or its likelihood of interfering with the pathogen (Table 2). In the first case, microbes from the same habitat (phyllosphere or rhizosphere) and the same or similar host species (especially where disease is absent; e.g., suppressive soils) would seem to be the best prospects. The rationale for this is that sustained biocontrol is likely to require that the organism be able to colonize (i.e., to adhere and grow). Resident organisms (those indigenous to a particular habitat) are presumably better adapted to them in general than are introduced or exotic organisms. However, this generality requires several qualifications. A non-resident might be induced to colonize if properly formulated (or if the host were modified genetically to make it more hospitable). Also, there is little evidence for host plant specificity among epiphytes or endophytes, other than those involved in mutualistic symbioses (Harley & Smith 1983). Finally, at an even more general level, many microorganisms seem to be undemanding with respect to substratum (although in some cases habitat preferential strains might exist). For example, *Aureobasidium pullulans* is a ubiquitous species (Cooke 1959), having been isolated from almost every conceivable habitat except the deep oceans. Many human pathogens have niches elsewhere: the fungus *Sporothrix schenckii*, which causes skin lesions, is associated with vegetation, including sphagnum moss (D'Alessio et al. 1965). The bacterium *Legionella pneumophila*, cause of the famous "Legionnaires disease", grows in association with algae and aquatic plants (Tison et al. 1980). Success of the so-called "classical" insect biocontrol programs based on importation of natural enemies, and the occasional spectacular invasions of exotic plant pathogens, weeds, or insects further

testify that being indigenous to a particular community is not a requirement for "success". Thus, while focusing on more prospective sources (in the upper portions of Table 1), we should no discount microbes of other environmental or animal (skin, gut) origin.

In Table 2, microbe sources are ranked in descending order of presumed effectiveness based on their likely armament potential as antagonists. For example, it is more than coincidental that several genera or species have repeatedly shown antagonistic potential. These include *Trichoderma, Chaetomium, Coniothyrium, Penicillium, Gliocladium, Trichothecium, Bacillus, Streptomyces,* and *Pseudomonas*. Hence, screens might effectively be designed to concentrate on, or enrich for, such organisms. Another profitable route might be to search for isolates that are taxonomically related to the pathogen (hence, could be expected to have at least some ecological similarity which could be used to advantage). For instance, epiphytic species of *Fusarium* or *Pseudomonas* might be more likely to have biocontrol potential toward their respective congeneric pathogens than would completely unrelated taxa. Better yet, one could search for nonpathogenic strains. The extreme in this strategy of obtaining "niche overlap" would be to delete the pathogenicity genes, then use the disarmed pathogen as a biocontrol agent against the wild type.

Knowledge of the mode of parasitism can be of some value in predicting an effective type of antagonist. Biotrophic pathogens (e.g., the rusts) may require no exogenous nutrients for penetration yet be exposed on the leaf surface for sufficient time prior to penetration to be potentially vulnerable to antagonists which are hyperparasites or act by antibiosis (Blakeman 1988). In contrast, necrotrophs (*Alternaria, Botrytis*) tend to grow saprophytically on the phylloplane, taking up exogenous nutrients before they can penetrate. Antagonists operating as nutrient competitors could be effective in this latter situation but would be predicted to be ineffective against biotrophs (Blakeman 1988). However, basing

TABLE 1. Sources of biocontrol agentes based on presumed colonizing potential of the microbe.*

1. Same habitat (e.g., phylloplane); same host species (especially where no disease occurs).
2. Same habitat; different host species.
3. Different habitat (e.g., rhizoplane); same host species.
4. Different habitat; different host species.
5. Other "likely" sources (e.g., terrestrial environments).
6. "Unlikely" sources (e.g., aquatic environments; animal symbionts).

* Ranked in descending order of probable importance.

TABLE 2. Sources of biocontrol agentes based on presumed antagonistic potential of the microbe.*

1. Species or genus of known antagonists.
2. Same species or genus as pathogen.
3. Mode of antagonism compatible with biotrophic/necrotrophic mode of parasitism.
4. Species-rich communities.
5. Phenotypic properties correlated with antagonism.

* Ranked in descending order of probable importance.

a screen on presumed mode of parasitism has several limitations. Among these are: 1) the biotrophy/necrotrophy demarcation is not absolute, but rather exists as a continuum (Andrews 1975). In any case it has little relevance for antagonism of plant pathogenic bacteria (which in theory can be categorized as biotrophs or necrotrophs, but which infect by direct introduction or through openings); and 2) the mode of antagonism must be known in advance, it must operate in nature, and in practice probably few organisms antagonize by a single mechanism.

Species-rich communities of relatively high microbial biomass and productivity are probably shaped relatively more by biotic interactions than those that are less saturated. Microbes in the former habitat are more likely to be selected for competitive ability, e.g., to possess such traits as the ability to obtain food at very low food densities, and production of antibiotics (Andrews 1984). Thus, the tropical rain forests, where microbial layers 22 um thick may exist on leaf surfaces (Ruinen 1961), would seem to be better places to look for antagonists than would the arctic tundra.

Finally, if it is known, even if empirically so, that antagonists as a group share certain selectable properties, then an enrichment process exploiting such features can be devised. For example, evidently many soilborne antagonists are resistant to soil treated at relatively low temperatures by aerated steam (50-70°c for 30 min), whereas non-antagonists are killed (Baker & Cook 1974). Other procedures that can be used, often sequentially, to selectively eliminate or inhibit extraneous microbes include drying (of soil), selective chemicals or media (including the plant itself), and ionizing radiation or other physical factors.

Whatever the source of the microbe it is important that detailed records, regarding the source, be kept, and that cultures be stored properly to insure viability and minimize genetic changes (Kirsop & Snell 1984).

THE COMPONENTS OF A SCREEN

The options for screening are assays under controlled conditions, uncontrolled conditions, or both. The former, in turn, consist potentially of two components: evaluation on agar media (in petri dishes or on agar-coated microscope slides), and bioassays on plants (typically seedlings) in a growth chamber or greenhouse. Each has advantages and disadvantages (Table 3). Effective biocontrol involves several properties of an antagonist and results from a sequence of events. The various assays evaluate these different properties with varying degrees of adequacy. All assessments are based either on interference (antagonism) per se, or on reduction in disease.

Assay under controlled conditions

Evaluation in vitro on agar media. The basic agar plate test in one of its many forms is well known and was popularized several decades ago in the era of antibiotic research. Candidate microbes are matched in various ways with the target organism and activity is evaluated semi-quantitatively based on the extent of inhibition of growth of the pathogen (Andrews 1985).

The attractive features of this assay all emerge from the fact that it can be designed simply, conducted quickly, and interpreted easily. Hence, the test is amenable to mass screening of candidate microbes which can be compared readily by the relative diameters of inhibitory zones. The assay can be varied in straightforward fashion, e.g., by altering the medium, incubation temperature, or timing of plating of microbe and pathogen. The results are often striking and may be suggestive of the mode of antagonism (antibiosis and/or nutrient competition).

The major disadvantage of the agar plate assay is that traditionally there has been poor correlation between inhibition in vitro and field performance (Andrews et al. 1983; Wong

TABLE 3. Comparison of representative tests for screening antagonists.*

Conditions	Assay	Ecological condition	Variable mesured	Key considerations	
				Advantages	Disadvantages
Controlled	Agar plate	Complex usually nutritious substrate	Interference (growth; macroscopic)	Cheap, fast; Can see interactions; conditions easily varied; can compare strains; can test mechanism	Poor predictor of biocontrol; unnatural conditions
Controlled	Agarose slide	Defined, usually minimal, substrate	Interference (germination; microscopic)	Uniformity; closer simulation to actual conditions	Poor predictor of biocontrol; time-consuming
Controlled	Seedling	Possibly genetically variable plants	Interference & disease	Measures antagonist performance on plants; flexibility	Plant variability; poor predictor of survival in nature
Uncontrolled	Field microplot	Genetically uniform (clonal) plants	Interference disease	The definitive test	Expensive; time-consuming results may not be generalizable; may be too severe

* Modified from Andrews (1985).

& Baker 1984; Weller et al. 1985). Usually the error is one of false positives, but false negatives can also occur. The "white" yeasts may show no inhibitory properties in culture tests, yet be effective in controlling foliar disease in the field (Fokkema 1976). Some strains of fluorescent psuedomonads suppress the root disease take-all of wheat, yet show no inhibition in vitro (Weller et al. 1985). Any assay that does not involve plants would not detect an effect stemming from induced resistance.

Evaluation in vitro on agar slides. This is essentially a microscopic version of the plate assay (Table 3) in which clean microscope slides are coated with nutrient deficient agar or agarose, spread with a mixture of pathogen and antagonist propagules, incubated, and the effects on germination and morphology of the pathogen recorded (Andrews 1985). Results are available sooner than is the case with the plate assay and interactions can be observed on a scale that would be difficult or impossible to devise with a petri dish. However, examinations are much more time-consuming. We found that data on germination and germ tube growth from agar slides corresponded well with that obtained directly from leaf surfaces, but that neither assay correlated well with effect on disease severity (Andrews et al. 1983).

Evaluation on plants. In this assay, typically small plants (frequently open-pollinated seedlings of species normally propagated clonally for agricultural purposes)

are inoculated with the pathogen concurrently with, or before or after, inoculation with the candidate microbe. Some measure of disease severity is later recorded, often fairly subjectively. Note, then, that an interaction phenotype among pathogen, plant, and antagonist is the main basis for the assessment, and what is measured is **biocontrol potential**. This integrates many properties of an organism (e.g., ability to scavenge nutrients; growth rate; colonizing potential; production of inhibitory compounds). The assay is therefore unlike in vitro tests (above) which quantify only interference or **antagonistic potential**, and are usually a function of only one or at most a few properties of an organism. A good antagonist can thus be a poor biocontrol agent.

The plant/controlled environment assay is the most critical sort of test in a screen because it represents the best compromise between the standardized, reproducible, but highly artificial conditions of the in vitro assays (above) and the realistic, but variable and logistically demanding field assays (below). Hence, it has many advantages (Table 3). Few false negatives would be expected because it is highly unlikely that an agent effective in the field would be ineffective under controlled conditions. However, as for the in vitro assays, there are many false positives, which are the reason for the poor correlation between these results and field performance. This is presumably because experimental designs to date do not adequately incorporate biotic and abiotic rigors of the natural environment.

There are other shortcomings or limitations with the plant/controlled environment assay. One is that because space is confined, researchers must use small plants. Apple seedlings or very small, grafted whips must be used in screens, not the standard tree! Small containers impose constraints on experiments designed to screen for biocontrol agents against soilborne pathogens. Typically, for example, seedlings are used rather than mature plants. Thus, the wrong developmental state of the plant may be used which may mean that the wrong conclusions are drawn. Another limitation is that while the environment can be well controlled it is rarely, if ever, clear what is the relevant environment for the assay!

It should be possible to better simulate environmental stresses (e.g., by applying natural microflora; using natural field soils or exposing plants to air spora; simulating key environmental variables). At the cost of imparting more realism (fewer false positives), however, these manipulations will convert a controlled environment assay with a high and relatively constant signal:noise ratio to one of lower and variable signal:noise ratio. Ultimately, there is a trade-off; the goal should be to incorporate sufficient **meaningful** (biologically relevant) variation into a logistically feasible assay without swamping the signal (i.e. biocontrol potential). One way of achieving both realism and reproducibility is to search for standardized tests that are good predictors of biocontrol activity in the field. These tests might not necessarily even involve plants, and they may well be found empirically or by chance. The assays could be for such attributes as colonizing potential and metabolite production under specific environmental regimens. However, it is noteworthy that to date there has not been a good correlation betwen obvious phenotypic features and epiphytic fitness. It may be possible with automated methods to screen large numbers of organisms concurrently for many attributes (e.g., substrate utilization pattern; colonizing ability; secondary metabolites) and to compare the profile of such organisms with known biocontrol agents for closeness of match. Eventually it may even be possible to screen for key molecules by use of rapid biochemical tests (e.g., ELISA) or, if the nucleotide sequences for key phenotypic properties are known, to search for these by molecular probes.

Assay under uncontrolled conditions

Field assays are of course the definitive test

of a candidate biocontrol agent which few have passed. The most noteworthy successes are *Agrobacterium tumefaciens* and *Peniophora gigantea*. Early success evidently is often due to fortuitous circumstances (Boudreau & Andrews 1987) and usually has been followed if not by failure then by inconsistent performance. For various reasons, failures are rarely pursued to determine the cause or possible remedy.

The main challenge with field tests is to devise a logistically feasible assay that is reproducible. In other words, the struggle is to achieve both realism and generality. Any field results are realistic in that they obviously apply at least to the actual test conditions in nature. However, they are not necessarily generalizable to other sites and times. So, ungeneralizable results and logistical impossibility are at the opposite ends of the same continuum. Improvement in one comes at some cost in the other. Logistics can be improved by use of micro-plots (individual plants or plant parts such as branches or leaves), but if the scale drops too far, this compromise is effectively meaningless.

Field tests may be too severe and result in potential biocontrol agents being abandoned prematurely. For example, such tests may be conducted under extreme conditions of inoculum pressure. A more common situation, however, is the issue of fitness in nature of an organism raised in the laboratory. The disparity between results of tests under controlled conditions and those under uncontrolled (field) conditions quite possibly reflects inappropriate conditions for mass culture and formulation. For this reason it is worthwhile to investigate the cause for failure in the field (i.e., to determine whether it is lack of colonization, or lack of antagonism, or both) and also is why a controlled environment assay should be retained as part of any screening protocol (see next section). Ideally, formulation issues should be addressed, along with technical and economic feasibility, before the candidate is screened in the field. Unfortunately university researchers rarely have the resources to pursue these considerations, but they can be undertaken by industry.

The culture and formulation issue presents the investigator with innumerable choices and is yet another place where compromises have to be made. Should one take fewer candidates and invest the saved resources by growing them under various conditions (e.g., solid surface vs. liquid fermentation; media varying in chemical composition) and applying them in various ways (e.g. propagule type; stage of growth; time of application; type of amendment)? Or, is it better to use a standardized, streamlined protocol that allows more agents can be screened? There are no easy answers to these questions, but undoubtedly a major focus of research in the coming decade should be on formulation.

SEQUENCE OF TESTS

A proposed screen extended from that suggested earlier (Andrews 1985) appears in Figure 1. This screen is not really new but is rather intended mainly to formalize or codify procedures used to varying degrees by different researchers. The common entry point for all candidates from sources outlined in Tables 1 and 2 is the controlled environment assay (top). Screening is concurrent by an *in vitro* method (A = agar plates or agarose slides) and by an *in vivo* procedure (P = plants in the greenhouse or growth chamber). The fate of each candidate is determined by the four resulting permutations of activity. The A+P- organism is stored for future consideration, possibly as a source for useful antagonism genes. The A-P+ and the A+P+ candidates are carried forward to the uncontrolled environment assay (Fig. 1, bottom). Any microbe positive in the field through successive sites and years enters the scale-up process (storage and formulation considerations; toxicity tests; registration procedures, etc) for commercial production. Organisms negative at this step should not be

discarded without some examination of the reasons for failure in the field. Then, if appropriate, corrective measures, such as reformulation, can be taken. If these fail or are deemed unwarranted, this candidate is also stored for possible future consideration.

In principle, this screen is applicable regardless of permutations (e.g., concurrent assay of several microbes for synergistic activity) that might be incorporated.

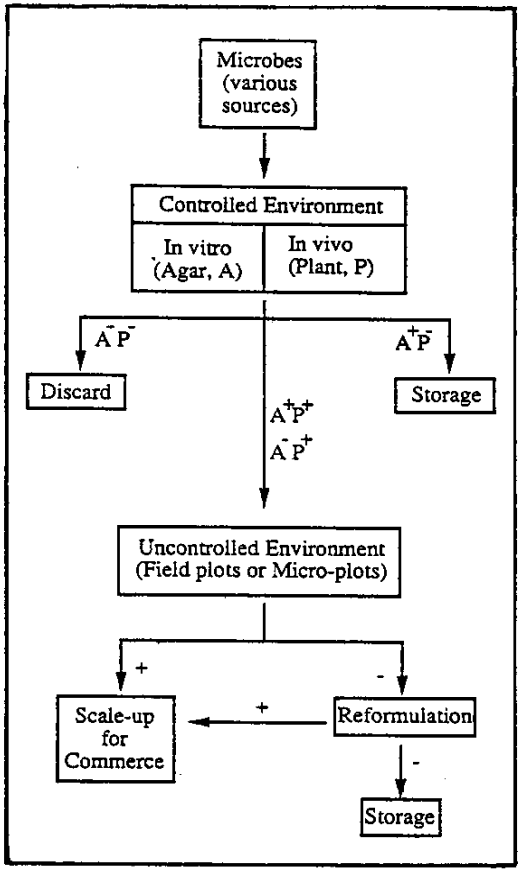

FIG. 1. Proposed components and sequence of assays in a screen for biocontrol activity. + designates activity (antagonism or disease suppression); - designates no evidence of activity.

CONCLUSION

Two kinds of errors can occur in efforts to select biocontrol agents from the countless species and strains of microorganisms available. First, a potentially good agent may be mistakenly discarded. This is analogous to the Type I error of statistics (rejecting the null hypothesis when it is true). Second, an ineffective microbe may be retained. This is analogous to the Type II error of statistics (accepting the null hypothesis when it is false). The goal of any screening procedure should be to minimize the probability of these two outcomes (Andrews 1985). An efficient or optimal screen is one that will accomplish this at the lowest unit cost of resources expended.

The continuing dilemma facing all biocontrol researchers is that, on one hand, in vitro assays – while providing for cheap, mass screening of candidates – are poor predictors of field performance (false positives). On the other hand, field assays – while realistic and potentially generalizable – are logistically impractical for mass screening and may be too severe (false negatives). Designing a screen is further complicated by the fact that we still have little knowledge of the phenotypic features that determine "success" as a biocontrol agent. Finally, it is important to realize that any screen may be valid only for those conditions imposed by the protocol. Thus, changing the variables such as media or temperature in the laboratory, or the time or place of field assays may produce very different results.

Given our sparse knowledge about growth and formulation of microbes for competence *in vivo*, I do not believe that any screen should consist only of a field assay, even if the logistics issue could be overcome. Therefore, one is forced to accept some kind of preliminary assay under controlled conditions.

The screen proposed in Figure 1 should provide both for good resolution of detection and good efficiency. Like all categorization schemes it is simplistic and considerable judgement has to be used, for instance, in

deciding what level of activity warrants retention versus rejection of a candidate. If anything, the screen may be overly conservative. Inclusion of agar assays concurrently with screening on plants (controlled conditions) allows for four rather than two permutations: with plants alone a positive response would lead to retention (for further assay in the field); a negative response would lead to rejection. Note that where the assay on plants is (+), the reaction on agar is inconsequential as to the fate of the candidate (an A- organism is carried further anyway; an A+ only reinforces the decision to proceed). However, a putative mechanism (antibiosis or nutrient competition) may be revealed for further testing. But the real advantage of the agar test is where the plant assay is negative. Instead of being discarded, as would be the case if only plants were used, the organism reacting positively on agar is stored for reconsideration.

Eventually assays at the controlled environment or field level may be designed that are manageable and good predictors of biocontrol performance. Until this is accomplished, a scheme such as the one proposed here appears to be the best compromise, at least as a point of departure in designing screens which may ultimately have to be case-specific.

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