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Microbial selection strategies that enhance the likelihood of developing commercial biological control products

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Research interest in utilizing microorganisms to create a microbial environment suppressive to plant pathogens has increased exponentially in recent years. Despite intense interest in developing biological control agents, relatively few antagonists have achieved 'commercial product' status. The fact that such a small proportion of active laboratory antagonists are developed into biological control products is partly due to several features common to microbial selection strategies that are widely utilized to obtain putative biological control agents: (a) relatively few candidate microorganisms are tested; (b) microbes are selected based on the results of an assay that does not replicate field conditions; and (c) the amenability of microbes to commercial development is excluded as a selection criterion. Selection strategies that enhance the likelihood of developing commercial biological control products are described. These include making appropriate choices regarding the pathosystem for biological control, the method of microbe isolation, and the method of isolate characterization and performance evaluation. A model system of developing a biological control product active against *Gibberella pulicaris* (Fries) Sacc. (anamorph: *Fusarium sambucinum* Fuckel), the primary causal agent of Fusarium dry rot of stored potatoes, is used to illustrate the proposed selection strategy concepts. The crucial importance and methodology is described, of selecting strains with enhanced potential for commercial development based on a strain exhibiting both favorable growth kinetics and bioefficacy when grown in commercially feasible liquid media.

Keywords: biological control; microbial selection strategies

Introduction

In 1987, Dr Kenneth F Baker [2] documented a sharp increase in research activity on the biological control of plant pathogens. He noted that from 1960 to 1981, the number of papers published per year on the topic rose from approximately 15 to nearly 200. The publication rate of papers on the biological control of plant pathogens has been maintained and likely exceeded in subsequent years. This level of research interest has generated a wealth of information on the entire spectrum of factors that influence the ecology, and ultimately the impact, of microorganisms on biotic causative agents of plant disease. However, though the disease-controlling activity of hundreds if not thousands of microbial strains has been described in publications, only 17 agents for the biological control of plant diseases are currently registered and sold in the United States [11]. Furthermore, as of 1994, chemical pesticides still accounted for approximately 98% of all pesticides sold in the world [33].

Many factors account for why there is a preponderance of biological control demonstrations in laboratory environments, yet a relative scarcity of commercially available products. These factors include but are not limited to: unfavorable economics of biomass production or market size; biomass stabilization or formulation difficulties; lack of patent protection of a potential product formulation or active ingredient; inconsistent product performance due to insufficient knowledge of the ecology of the antagonist, target pathogen and associated microflora; and cost-prohibitive registration expenses.

A less commonly considered but important factor in the infrequent commercialization of laboratory active biological control agents is that many of these agents were selected using improperly conceived microbial selection strategies. With few exceptions, studies in public sector research are not initiated and microbial selection and evaluation strategies are not devised with the goal of developing a commercially feasible biological control product. Thus, many opportunities to select for a microbial agent that possesses attributes that enhance its likelihood for development as a marketable product are missed. A poorly conceived microbial selection strategy is a costly mistake since it occurs near the beginning of a very long, arduous process of developing a biological control product.

In this article, we have divided strategies for discovering antagonists with enhanced potential for commercial development into three broad categories: (1) choosing an appropriate pathosystem to investigate; (2) choosing an appropriate method of microbe isolation; and (3) conducting an appropriate isolate characterization and performance evaluation. For purposes of illustration, we will rely heavily on our research experience [25,26,29] on selecting bacterial strains for the biological control of Fusarium dry rot, an important postharvest disease of potatoes [3–5,13]. However, the majority of the strategies described for selecting

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biological control strains with enhanced potential for commercial development are applicable to any pathosystem targeted for biological control.

Choosing an appropriate pathosystem (Figure 1)

Choosing an appropriate pathosystem to investigate may seem to be an unusual selection strategy, but if you have the option, your choice of a pathosystem for biological control will have a huge impact on developing a biological control product. For instance, the chance of successfully developing a biological control product is increased if one chooses to work on a pathosystem where the etiology of the plant pathogen has an obvious weakness, or where the pathogen operates in a microenvironment favorable to introduced antagonists. When deciding on a pathosystem to attempt to biologically control, we chose to develop microbial agents active against Fusarium dry rot (primary causal agent, *Gibberella pulicaris* (Fr.:Fr.) Sacc. (anamorph: *Fusarium sambucinum* Fuckel)) in stored potato tubers. A major weakness of the etiology of this pathogen is that it requires a wound in order to infect and tubers are able to heal wounds, under proper storage conditions, in less than 2 weeks [15]. Additionally, the pathogen operates in an environment that is favorable to introduced antagonists in that tuber storage temperatures are uniform and relative humidities are high (90% or higher), a feature true for many postharvest pathosystems. Soils with reduced microbial diversity or content, such as can be the case with nursery potting mixes that are pasteurized or solarized field soils, can also be appropriate choices for developing and applying biological control agents.

Pathosystems that do not have readily available control options can be good targets for the development of biological control agents. This would be the case, for instance, if chemical controls are unsuitable due to resistance in the pathogen population or potential problems with chemical residues. Thiabendazole resistance in field populations of *G. pulicaris* [7,13,14,17,27,31] and low tolerances for chemical residues on harvested commodities again supported targeting Fusarium dry rot for biological control. Furthermore, high levels of resistance are not readily obtained from potato breeding stocks [20]. Finally, it should be noted that the development of a biological control product is expensive, as is its final production. A pathogen chosen for potential control with a biotic agent should be one that is economically damaging enough to justify the economic risk a company would take in product development. Such is the case with *G. pulicaris* on stored potato tubers where the pathogen is estimated to cause annual storage losses of 6% to as high as 25% [5], which would represent a loss of at least 100 million dollars per year for United States producers alone. In addition to destroying tuber tissues, *G. pulicaris* can produce trichothecene toxins that have been implicated in mycotoxicoses of humans and animals [8,28], thus adding to the economic significance of this disease.

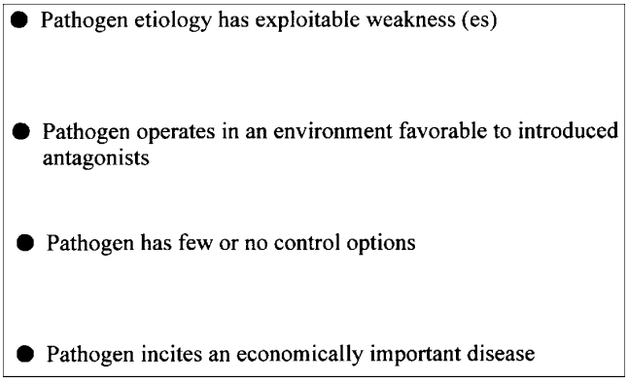

Figure 1 Pathosystem characteristics that enhance the likelihood of developing a commercial microbial product for controlling the disease.

Choosing an appropriate method of microbe isolation (Figure 2)

In their classic 1974 book on biological control of plant pathogens, Baker and Cook [1] stated that, 'antagonists should be sought in areas where the disease…does not occur, has declined, or cannot develop, despite the presence of a susceptible host…' This insightful statement is as apropos today as when it was originally made more than 20 years ago. In addition to adhering to this important first step, microbial isolation techniques designed to increase the likelihood of developing a commercial biological control product should incorporate the following concepts: maximize the number of microbes considered, isolate from appropriate plant parts, isolate under appropriate environmental conditions, and avoid the use of highly selective media.

The importance of searching for prospective biological control agents in areas with little disease development, as described by Baker and Cook [1], is based on the assumption that a more productive search for biocontrol agents will occur in areas where biological control is naturally occurring in the field, as opposed to areas where it is not. For instance, studies based on this premise led to the discovery of many effective biological control agents including strains of *Pseudomonas* spp active against take-all of wheat [32]. Based on probability alone, evaluating a maximal number of putative biocontrol agents increases the chance

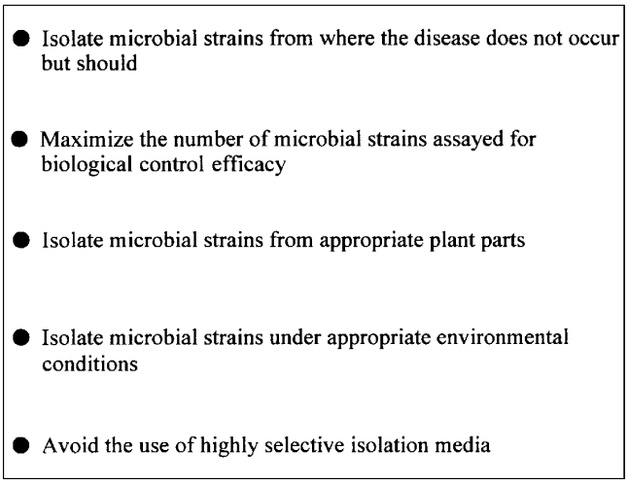

Figure 2 Microbial isolation methods that enhance the likelihood of discovering strains with high commercial development potential.

of discovering a particularly effective strain. Isolating prospective biological control agents from the specific plant tissues that require protection and under realistic environmental conditions helps insure that antagonists isolated will be well adapted to survival and activity on the specific plant part or parts requiring protection in the field. We attempted to incorporate all of these microbe isolation concepts into our work to develop biological control agents for the control of Fusarium dry rot. Our techniques are diagrammatically summarized, including the screening of individual isolates for biological control efficacy and the selection of 18 bacterial 'finalists,' in Figure 3. Briefly, our microbe isolation method was as follows. Gamma irradiation-sterilized field soil samples were enriched with potato tuber periderm, inoculated with a small amount of field soil obtained from potato fields with little dry rot disease incidence and incubated for 1 week at 15°C. The microorganisms most adept at rapid growth at 15°C on the nutrients found in potato periderm and at wound sites, therefore, made up the majority of microbes in each recolonized soil sample. In our studies, conidia of *G. pulicaris* were then added to the microbially recolonized soils, and 2 days later, aqueous soil pastes of each soil were applied to wounded potato tubers. Whole tubers were used in our assays rather than potato slices in order to maintain potentially critical interactions between intact host defense systems of whole tubers, putative biocontrol agents and *G. pulicaris*. After incubation for 4 weeks at 15°C, tubers were scored for dry rot disease development. Those wounds that developed inconsequential disease were highly likely to contain microbial communities that, in concert, suppressed disease development. Furthermore, suppressive microbial communities present in disease-free wounds exhibited suppressiveness on the specific plant part (wounds) and under environmental conditions (high relative humidity, 15°C) where a microbial strain sold as a biological control product would be expected to perform in the field. It should be noted that the assay also assured that any suppression identified was biological in nature since 95% of each test soil was chemically, physically and nutritionally identical, with the only substantive difference being the microbial content supplied by the various field soil samples. Because only treatments that developed inconsequential disease were chosen for the labor-intensive step of isolating individual microbial strains, whole microbial populations representing thousands if not tens of thousands of strains of microorganisms did not have to be individually purified and assayed for biological control capability. By using this strategy of screening for putative biological control agents, the effectiveness of theoretically hundreds of thousands of organisms can be screened and organisms isolated only from those soils that contain microbial strains capable of exhibiting biological control singly or in concert with other strains.

The isolation of individual microbial strains should be accomplished on media that favor bacteria, fungi, actinomycetes and yeasts, so as to insure representatives of each microbial taxon are recovered for testing. However, exclusive use of media that are more selective in nature, such as media that select only for Pseudomonad bacteria for instance, should be avoided. Otherwise, potentially fruitful microbial groups that do not grow on the selective media used may be missed. Even the exclusive selection of spore-forming bacteria, on the basis that these organisms more readily survive drying than do Gram-negative bacteria, is difficult to justify given recent advances in the stabilization of Gram-negative bacteria. A good compromise medium to use for microbial recovery would be one that is a solidified version of a commercially feasible liquid medium, such as one that is composed of inexpensive nutrient sources (distiller solubles, corn steep liquors, etc). Thus, even at this early stage of biological control product development, the researcher would potentially be preselecting for strains exhibiting the prerequisite trait of growth on a commercially feasible medium while selecting against strains that are not commercially viable choices. The issue of liquid culture production of biological control agents is discussed in more detail later.

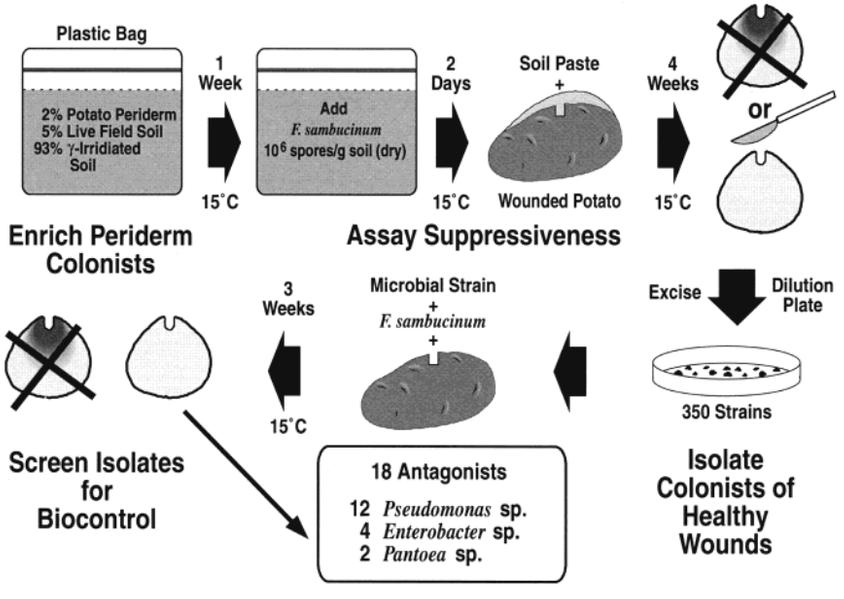

Figure 3 Isolation of microbial antagonists effective in suppressing Fusarium dry rot of potatoes [25].

Conducting an appropriate isolate characterization and performance evaluation (Figure 4)

Avoid in vitro Petri plate antagonism tests

The first issue of importance in conducting appropriate isolate characterization and performance evaluation is to avoid the use of *in vitro* Petri plate antagonism tests as a method of selecting strains for further testing. As defined here, Petri plate antagonism tests screen for reduced growth of a plant pathogen on a solidified medium when the pathogen is in the vicinity of a putative antagonist growing in the same plate. The reason for avoiding this antagonist selection method is clear; results from Petri plate antagonism tests invariably do not correlate with *in situ* biological control results [10,18,21,22]. Additionally, virtually every change in the method of conducting such tests can dramatically change the outcome of the test [9,12,24]. Equally disconcerting is that antagonists capable of controlling disease via competition, induced disease resistance and (frequently) mycoparasitism are not detected by Petri plate antagonism tests. Only if a particular biological control system is so well characterized that the predominant mode of action of successful biological control agents is known, can Petri plate selection for microbes possessing that attribute be considered.

Develop a realistic bioassay

The next step in conducting appropriate isolate characterization is to develop a realistic bioassay. If Petri plate antagonism tests are not realistic or reliable, then how can large numbers of putative biological control agents be accurately reduced to a more manageable number of strains, each with efficacy in controlling disease? At this stage, we suggest it is most appropriate to develop and use a bioassay that closely mimics the field environment (climatological, microbial, physical, chemical) to insure results of bioassays are as predictive as possible of results that would be obtained in the field. This means developing a bioassay that tests putative antagonists on whole plants grown in field soil or on a harvested commodity under environmental conditions that mimic field conditions. This type of bioassay not only will produce results with the highest likelihood of being predictive of strain field performance, but enables discovery of candidates that should be eliminated from consideration based on their deleterious impact on intact host tissues. Single replicate tests using the bioassay developed and requiring a candidate strain to demonstrate an extremely high level of biological control will insure that only the very best candidate strains are carried onto further tests. A realistic bioassay will also have the timing of the arrival of the pathogen and antagonist inoculum the same as it would be for utilizing a biological control product. Thus, ideally, if one is attempting to discover microbial agents for controlling postharvest pathogens, the bioassay will have the pathogen inoculum present on the host before the antagonist product arrives. For antagonists that can be applied to seed, the bioassay should have the antagonists coated on seed prior to being exposed to the pathogen (assuming the pathogen is not seed borne). A final consideration for developing a realistic bioassay is that pathogen inoculum for the bioassay should be produced utilizing nutrient media that have nutrient balances and concentrations that approximate those under which pathogen inoculum is naturally produced. This is important because nutrient balance and concentration can greatly influence the characteristics of the inoculum produced. The C:N ratio of media, for instance, can influence the morphology and efficacy of conidia of *Colletotrichum truncatum* [16,23]. Lack of attention to media composition when producing pathogen inoculum for bioassays could decrease the accuracy of a bioassay in selecting top biological control candidates.

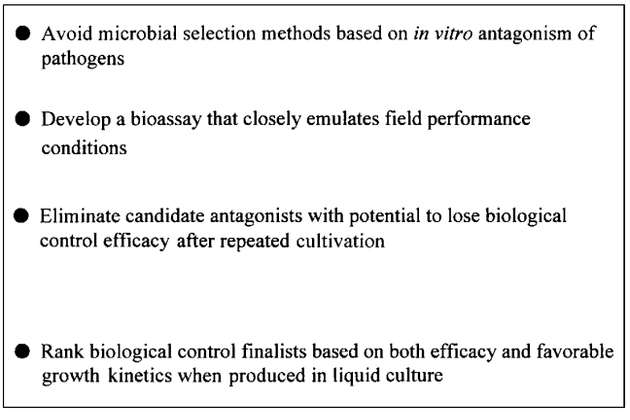

Figure 4 Microbial isolate characterization and performance evaluation methods that increase the possibility of selecting strains with enhanced commercial development potential.

Consider the genetic stability of antagonists

After utilizing an appropriate bioassay to drastically reduce the number of microbes considered for further tests, a potentially fruitful next step is to reduce the risk of selecting finalists that would readily lose biological control efficacy upon repeated laboratory cultivation. Multiple generations of a microbe will be produced if the microbe is developed as a biological control product and thus, instability of genes involved in biological control efficacy would render the candidate unacceptable for product development. All candidate strains should therefore be subcultured numerous times prior to ranking finalists. Strains that were efficaceous but lose efficacy after repeated cultivation will not rank high in subsequent multiple replication bioassay tests (described below) and will be dropped from further consideration. It should be noted, however, that strains that maintain bioefficacy after repeated subculturing may still demonstrate bioefficacy instability when subjected to the increased rigors of industrial scale fermentation and formulation. Ranking antagonist finalists using an objective system such as two-dimensional liquid-culture focusing (described below) will insure that the most suitable replacement strains are known if this or other unforseen developments cause the top strain to be dropped from consideration for commercial development.

Rank finalists based on two-dimensional liquid-culture focusing

At this stage, virtually all researchers, at least as reported in the public sector, conduct further bioassays with antagonist

inoculum produced on solid media, choose their 'best candidates' and begin the extremely labor-intensive and expensive steps of conducting field tests and devising microbial formulation and stabilization techniques. Additional studies can include ecological studies of microbial interactions of antagonist with resident microflora, taxonomic characterization, colonization studies, and interactions of the 'best' antagonists with commonly utilized pesticides. The cost in time and dollars can be enormous. Unfortunately, this work is frequently conducted on a candidate strain that would be difficult to produce in liquid culture or is not, in fact, the most efficacious strain. We submit that the process of choosing the best candidate from the list of finalists should be completed using a process of 'two-dimensional liquid culture focusing.' We define two-dimensional liquid culture focusing as utilizing microbial antagonist attributes of efficacy in controlling disease when produced in liquid culture and amenability to production in liquid culture to rank the commercial development potential of putative biological control agents (Figure 5). Virtually every biological control paper in print ignores this crucial fact: not all microorganisms grow well in liquid culture or are adequately efficaceous when grown in liquid culture. Industrially important microorganisms are traditionally cultivated in stirred-tank fermentors charged with liquid media. Thus, if a biological control product is to result from research designed to discover antagonists, one must specifically rank antagonists based on efficacy and favorable growth kinetics when produced in liquid culture. Leaving such tests as an afterthought to take place after an enormous amount of work and time developing an agent to the point of scale-up, can result in a late discovery that your 'best' antagonist is not suitable for commercial product development due to poor efficacy or growth kinetics when grown in liquid culture.

Examples from our work with discovering biological control agents effective against Fusarium dry rot illustrate the importance of conducting two-dimensional liquid culture focusing [29]. First, the importance of ranking strain efficacy when the strain is produced in liquid rather than solid media is shown in Table 1. Of 20 putative biological control strains studied (18 strains selected using whole tuber bioassays as described earlier, plus two additional biological control strains from other sources), strain S09:Y:08 ranked 14th of 20 strains in biological control efficacy when produced on a solidified medium (1/5 strength tryptic soy agar). In traditional biological control selection processes, this strain would not have been considered further. However, when produced on commercially feasible minimal (MDL) and undefined (UDL) liquid media, the strain ranked second of 20 strains. Conversely, strains S11:T:04, S09:T:14, and S09:P:06 tied for highest efficacy ranking when produced on the solid medium and would have been selected for further study if traditional solidified media had been used for inoculum production. Such a selection would have jeopardized the potential of a commercially viable biological control agent resulting from the project, since these strains sometimes performed poorly and were never among the best strains when produced in liquid culture. Thus, to accurately evaluate which candidate antagonist strain is most efficaceous in controlling plant disease, one must rank the strains based on efficacy when grown in a liquid medium that emulates a commercial production medium.

Similarly, it is important to rank strains based on their amenability to production in commercially feasible liquid media since strains vary greatly in this attribute (Table 2). When grown in liquid media, strain S09:Y:08 achieved some of the highest volumetric biomass productivity (P) and maximum absorbance (A_{max}) values of any strain regardless of the liquid medium tested. The importance of evaluating strain amenability to liquid culture production on a variety of media is seen with strain S11:T:04 which had a high P when grown on UDL medium but one of the worst P values of all strains when grown on MDL medium.

In order to rank strains based on both bioefficacy and growth kinetics when grown in liquid culture, relative performance indices (RPIs) were calculated for each strain based on each attribute ($RPI_{efficacy}$ and $RPI_{kinetics}$). Each strain's overall RPI was calculated by averaging the $RPI_{efficacy}$ and $RPI_{kinetics}$ values. Overall RPIs were then used to objectively rank the strains' commercial development potentials (Table 3). RPIs are dimensionless indices that are between 0 and 100 when data are normally distributed. $RPI_{kinetics}$ is calculated for each strain as $RPI_{kinetics} = \{[(x - \bar{x})/\sigma] + 2\} * 25$, where x is a single observation value for a strain, $\bar{x}$ is an average of all observations from all strains being ranked, and σ is the standard deviation of all obser-

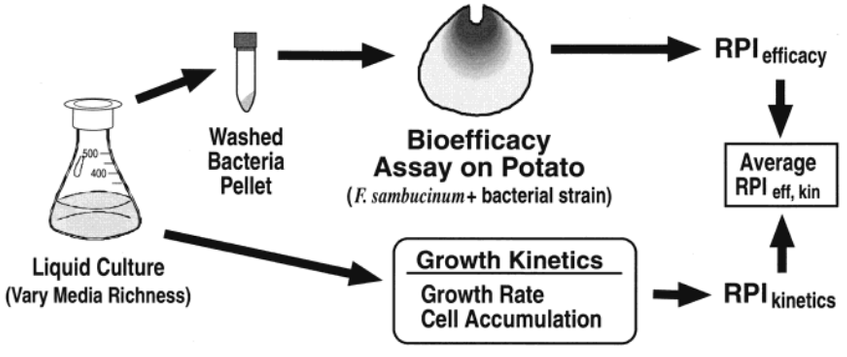

Figure 5 Two-dimensional liquid culture focusing method of objectively ranking the commercial development potentials of microbial strains based on relative performance indices (RPI) calculated from the strains' growth kinetics and efficacy when produced in liquid culture. RPIs are dimensionless indices that are between 0 and 100 when data are normally distributed. See text for formulas used to calculate $RPI_{kinetics}$ and $RPI_{efficacy}$.

Table 1 Relative efficacies of bacterial antagonist strains used for biological control of Fusarium dry rot as influenced by the cell production medium[1]

Bacterial strain	NRRL number	Disease rating (mm)			Ranking by efficacy		
		TSBA/5[2,3]	MDL[2]	UDL[2]	TSBA/5	MDL	UDL
S09:Y:08	B-21128	2.7	1.7	1.1	14	2	2
S11:P:14	B-21134	2.9	3.2	1.7	16	11	4
P22:Y:05	B-21053	2.0b	3.0a,b	5.2a	6	10	16
S11:T:04	B-21048	1.7b	4.9a	5.7a	1	13	17
S09:T:14	B-21051	1.7b	4.4a	3.2a,b	1	12	12
S09:P:06	B-21049	1.7c	5.2b	27.1a	1	16	20

[1]Selected results from 20 strains studied in Slininger *et al* [29].
[2]TSBA/5 = 1/5-strength tryptic soy broth agar; MDL = minimal defined liquid medium; UDL = undefined liquid medium.
[3]Within rows, disease ratings marked with different letters are significantly different ($P \leqslant 0.05$). Each disease rating is the average of two trials of four replicates each. 'Pathogen only control' disease rating, $\bar{x} = 26.6$ mm.

Table 2 Variation in liquid culture cell production rate and accumulation with bacterial strain and nutritional environment

Bacterial strain	NRRL number	Kinetic parameters[1]			
		P ($g\,L^{-1}\,h^{-1}$)		A_{max}	
		MDL	UDL	MDL	UDL
S09:Y:08	B-21128	0.06	0.09	19.2	16.1
S11:P:14	B-21134	0.05	0.11	15.7	15.9
P22:Y:05	B-21053	0.03	0.11	8.2	15.0
S11:T:04	B-21048	0.01	0.17	1.5	10.9
S09:T:14	B-21051	0.03	0.03	6.8	8.0
S09:P:06	B-21049	0.04	0.08	6.4	6.5
	Average of all strains	0.04 a[2]	0.10 b	8.9 a	13.9 b

[1]Selected results from 20 strains studied in Slininger *et al* [29].
[2]Different letters indicate average parameter values, measured on MDL (minimal defined liquid medium) and UDL (undefined liquid medium), differ ($P \leqslant 0.05$).

Table 3 Use of relative performance indices (RPI) to achieve a 2-dimensional assessment of antagonist commercial potential based on growth and efficacy of liquid-grown cells[1]

Bacterial strain	NRRL number	RPI[2]		Overall[3] $RPI_{eff,kin}$	Commercial[3] potential group
		Efficacy	Growth kinetics		
S09:Y:08	B-21128	66.3	64.9	65.6 ± 5.6	A
S11:P:14	B-21134	66.3	60.3	63.3 ± 4.4	A
P22:Y:05	B-21053	59.2	54.2	56.7 ± 5.7	C
S11:T:04	B-21048	47.1	45.4	46.2 ± 11.1	DE
S09:T:14	B-21051	60.9	25.1	43.0 ± 12.2	E
S09:P:06	B-21049	31.2	23.8	27.5 ± 9.6	F

[1]Selected results from 20 strains studied in Slininger *et al* [29].
[2]$RPI_{efficacy}$ and $RPI_{kinetics}$ are averages of six RPI values from two productions of cells on minimal defined (MDL), semi-defined complete (SDCL) and undefined (UDL) liquid media.
[3]Two-tailed *t*-test determined the ±95% confidence intervals about overall $RPI_{efficacy,\ kinetics}$ values. Means not significantly different have the same 'commercial potential group' letter.

vations from all strains being ranked. $RPI_{efficacy}$ is calculated as $|\{[(x - \bar{x})/\sigma] - 2\}| * 25$ since efficacy improves as disease rating decreases [29]. As seen in Table 3, some strains excelled in growth kinetics in liquid culture but not in efficacy while for other strains the converse was true. Strains that ranked high for both attributes, such as S11:P:14, were judged to have the highest commercial development potential and selected for the more labor-intensive steps required for biological control agent product development. The concept of using RPIs can be expanded to ranking additional strain attributes that would be considered favorable for commercial development. For example, survival and shelf-

life of strains subjected to commercially available dehydration procedures (such as air drying, freeze drying and spray drying) would be an additional attribute that could be worked into the RPI concept. The possibilities are extensive and could be tailored to meet the needs of the specific pathosystem and biological control product type required. Regardless, using the selection criteria of efficacy and growth kinetics when produced in liquid culture enables a rapid, objective ranking of strains prior to commencing the extremely time-consuming and expensive steps that remain to be completed in developing a commercial biological control product.

Conclusion

At this stage of the development of a biological control product, microbial selection has, in most respects, been completed since the two or three strains receiving the highest ranking from two-dimensional liquid culture focusing can all be taken onto the next stages of product development. Additional product development steps may include considering registration costs, conducting field tests, determining the mode of action, ecological studies, strain improvement, formulation studies, determining pesticide/biocontrol agent interactions, antagonist taxonomic classification (and related safety concerns, if any), and evaluating the possibility of broadening the product market potential via determining the efficacy of effective strains against other host pathogens. Which of these steps are appropriate to conduct next will depend on the specific pathosystem under study [6,19,30]. Regardless of which of these steps remain to be completed prior to achieving a commercial biological control product, the microbial selection strategies described in this paper take place in the first few months of a project but largely dictate whether additional years of research will result in a commercially available biological control product or an extensively researched laboratory phenomenon. A cornerstone of any microbial selection strategy should be the ranking of putative antagonists based on both favorable growth kinetics and bioefficacy when antagonists are grown in commercially feasible liquid media.

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