Combined Use of Biocontrol Agents to Manage Plant Diseases in Theory and Practice

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ABSTRACT

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Effective use of biological control agents (BCAs) is a potentially important component of sustainable agriculture. Recently, there has been an increasing interest among researchers in using combinations of BCAs to exploit potential synergistic effects among them. The methodology for investigating such synergistic effects was reviewed first and published results were then assessed for available evidence for synergy. Correct formulation of hypotheses based on the theoretical definition of independence (Bliss independence or Loewe additivity) and the subsequent statistical testing for the independence–synergistic–antagonistic interactions have rarely been carried out thus far in studies on biocontrol of plant diseases. Thus, caution must be taken when interpreting reported “synergistic” effects without assessing the original publications. Recent theoretical modeling work suggested that disease suppression from combined use of two BCAs was, in general, very similar to that achieved by the more efficacious one, indicating no synergistic but more likely antagonistic interactions. Only in 2% of the total 465 published treatments was there evidence for synergistic effects among BCAs. In the majority of the cases, antagonistic interactions among BCAs were indicated. Thus, both theoretical and experimental studies suggest that, in combined use of BCAs, antagonistic interactions among BCAs are more likely to occur than synergistic interactions. Several research strategies, including formulation of synergy hypotheses in relation to biocontrol mechanisms, are outlined to exploit microbial mixtures for uses in biocontrol of plant diseases.

Additional keywords: hypothesis testing.

Effective use of biological control agents (BCAs) is a potentially important component of sustainable agriculture. The principal biocontrol mechanisms involved include mycoparasitism, antibiosis, competition, and induced resistance (2,21,58); additional mechanisms are hypovirulence mediated through fungal viruses (70) and inhibition of enzymes involved in plant pathogenicity (42). Individual biocontrol mechanisms have been shown to be predominant for some BCAs but there are also many instances where more than one mechanism may operate in a given BCA isolate (20,77), and prolonged study of any individual BCA will often reveal additional biocontrol mechanisms. The ecological processes determining the success or otherwise of biological control are complex. Thus, it is not surprising that, in addition to variable control efficacies, biocontrol success in field crops has been limited despite much research effort. Most biocontrol success has been achieved in greenhouse cultivation (27,56), where ecological parameters are less variable. Because of the inconsistent or limited biocontrol achieved in the field, BCAs have also been used in combination with fungicides or cultural practices (22,24,64).

Use of mixtures of cultivars (54) or fungicides (5) has been successfully adopted in many crops to increase and maintain disease control efficacy when individual cultivars or fungicides may not be able to control disease effectively. To improve biocontrol efficacies achieved through use of a single BCA, there has been increasing interest recently among researchers in using mixtures of BCAs. A range of biocontrol mechanisms may operate in mixed BCA populations (23,33,34,72), although their relative significance is unknown but may vary with circumstances. In mixed BCA populations, we may need to consider both direct and indirect interactions between different BCA populations. Compared with the more efficacious BCA, combined use of two BCAs may lead to increased (48,79), reduced (4,25,74), or similar (16,38,74) biocontrol efficacy. However, in many such studies, the hypothesis testing for synergistic, additive, or antagonistic interactions among BCAs in controlling diseases was not clearly formulated and, thus, not tested statistically.

There is a need to assess statistical methodologies for investigating synergistic effects among BCAs and to evaluate the

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currently available evidence for their presence. This review is divided into three sections. The first section briefly reviews the statistical definition and testing of interactions. The second section summarizes recent theoretical modeling results on the effects of interactions among BCAs on disease development. Finally, we summarize results of experimental studies where combined use of several BCAs has been investigated and suggest possible research topics related to exploitation of microbial mixtures for biocontrol of plant diseases.

STATISTICAL DEFINITION AND TESTING OF INTERACTIONS

An explicit definition and subsequent testing of interactions are essential to communicate and compare research results from different studies. However, this is not a trivial exercise. Much effort has been spent on this topic in medical science relating to drug interactions (8,46,55,76,78). By comparison, an explicit statistical testing of a correctly formulated hypothesis on interactions among BCAs has been carried out in only a few studies of biocontrol of plant diseases.

Two methods are in common use for calculating the expected effects for the use of combinations of components as compared with individual components: Bliss independence (3) and Loewe additivity (51). These two methods yield different outcomes (Fig. 1), and only Loewe additivity correctly predicts the outcome in the simple case in which the two inhibitors are actually the same agent. For simplicity, we only discuss these methods as they apply to two BCAs acting alone or in combination but similar reasoning applies to three or more agents.

Bliss independence is also called effect multiplication or the fractional product; it also may sometimes be known as independent effect with overlap. It assumes that the two inhibitors act through independent mechanisms and, thus, combined use is represented as the union of two probabilistically independent events. The expected disease development (%) for the combination use (D_{12}) is computed as the product of those of two individual BCAs (D_1 and D_2) (i.e., $D_{12} = D_1 \times D_2$). If we assume the treatment efficacy is defined as $E_{12} = 1 - D_{12}$, then $1 - E_{12} = (1 - E_1) \times (1 - E_2)$. After algebraic simplification, this leads to the commonly used formula $E_{12} = E_1 + E_2 - (E_1 \times E_2)$, as used in

studies of synergy with fungicide mixtures (39) and biocontrol of plant diseases (34).

For example, if the control efficacy for two BCAs when used individually is 50% for both, then, under the Bliss independence, the expected efficacy is 75%. If the observed control efficacy (O_{12}) is greater than the expected (E_{12}), then synergistic interactions among the two agents are indicated. On the other hand, if $O_{12} < E_{12}$ then antagonistic interactions are indicated. Otherwise, no interactions (i.e., additive effect) are indicated.

Loewe additivity assumes that two inhibitors act through a similar mechanism, leading to the concept of dose substitution. Given the concentration of two inhibitors that individually achieve $X\%$ disease control ($[C_1]_X$, $[C_2]_X$), the concentration of inhibitors theoretically required to produce the same efficacy when used in combination ($[CI]_X$, $[CI_2]_X$) can be calculated as

$$1 = \frac{[CI_1]_X}{[C_1]_X} + \frac{[CI_2]_X}{[C_2]_X}$$

Loewe additivity involves dose-ratio addition and, therefore, is also called dose additivity.

A combination index (9) was developed to denote whether two inhibitors interact with each other:

$$\text{Combination index} = \frac{[CI_1]_X}{[C_1]_X} + \frac{[CI_2]_X}{[C_2]_X} \begin{cases} > 1 & \text{antagonism} \\ = 1 & \text{additivity} \\ < 1 & \text{synergy} \end{cases}$$

This index compares the doses of inhibitors individually and in combinations that experimentally produce the same level of inhibition. By definition, the nature of interaction among two agents may depend on the level of control efficacy. To use the combination index, a dose response is required for each agent.

THEORETICAL MODELING OF COMBINED USE OF BIOCONTROL AGENTS

A variety of approaches has been used to model plant-pathogen-BCA systems, ranging from simple infection-BCA dose relationships (6) to relatively complex simulation models (43,45). In some cases, biocontrol has been modeled with attention to factors such as energetic requirements for BCA growth (67) and spatial heterogeneity in mycoparasite distribution (41). Stochastic

FIGURE 1

Sample illustration of two methods used for testing synergistic or antagonistic interactions among two inhibitors.

A, Bliss independence, assuming the two inhibitors act through independent mechanisms and, thus, combined use is represented as the union of two probabilistically independent events.

BCA = biological control agent. **B**, Loewe additivity, assuming that two inhibitors act through a similar mechanism, leading to the concept of dose substitution. In B, three lines of the combination index for a control efficacy of 50% (ED_{50}) represent synergistic (dotted), additive (solid), and antagonistic (dashed) interactions, respectively, among two inhibitors. The precise shape of the combination index may depend on the control efficacy under consideration.

TABLE 1
Summary of experimental studies on combined use of biological control agents (BCAs)
to control plant diseases in comparison with the use of individual agents

Controlled organism	BCA1	BCA2	Host type	Number of treatments ^a				Ref ^b
				Total	>Pred	>Best	<Best	
<i>Colletotrichum acutatum</i>	<i>Cryptococcus laurentii</i>	<i>Metschnikowia pulcherrima</i>	Wounded apple	4	0	0	0	11
<i>Penicillium expansum</i>	...	...	...	4	0	0	0	...
<i>P. expansum</i>	...	...	...	4	0	0	0	10
<i>P. expansum</i>	<i>Pseudomonas syringae</i>	<i>Sporobolomyces roseus</i>	Wounded apple	4	2 (likely)	4	0	37
<i>P. expansum</i>	<i>Cryptococcus albidus</i> , <i>C. laurentii</i> , <i>Rhodotorula glutinis</i>		Wounded apple	4	1 (likely)	2 (likely)	1 (likely)	7
<i>Botrytis cinerea</i>	...	...	...	4	1 (likely)	1 (likely)	1 (likely)	...
<i>B. cinerea</i>	<i>Aureobasidium pullulans</i>	<i>R. glutinis</i>	Wounded apple	2	Unlikely	1	0	49
<i>B. cinerea</i>	Two <i>Bacillus subtilis</i> strains and <i>A. pullulans</i>		...	2	...	2	0	...
<i>P. expansum</i>	<i>A. pullulans</i>	<i>R. glutinis</i>	...	2	0	0	0	...
<i>P. expansum</i>	Two <i>Bacillus. subtilis</i> strains and <i>A. pullulans</i>		...	2	Unlikely	1	0	...
<i>P. malicorticis</i>	<i>A. pullulans</i>	<i>R. glutinis</i>	...	2	...	1	0	...
<i>P. malicorticis</i>	Two <i>B. subtilis</i> strains and <i>A. pullulans</i>		...	2	...	1	0	...
<i>P. expansum</i>	Yeast strains		Wounded apple	13	NA	4	NA	36
<i>Gaeumannomyces graminis</i> var. <i>tritici</i>	Several <i>Pseudomonas</i> spp. strains		Wheat seed	5	0	0	0	57
	...	...	Wheat seed	1 (chamber)	0	0	0	18
	<i>T. koningii</i>	<i>Pseudomonas</i> spp.	Wheat seed (<i>Pseudomonas</i>), soil (<i>T. koningii</i>)	6 (chamber)	0	0	0	17
	...	...	...	1 (field)	0	0	0	...
<i>Rhizoctonia solani</i>	<i>Trichoderma</i> spp.	<i>Bacillus</i> sp.	Cucumber seed	6	0	0	0	79
<i>Pythium ultimum</i>	Several <i>Pseudomonas</i> spp. strains		Sugar beet seed	7	0	0	5	29
<i>P. ulmum</i>	<i>Trichoderma virens</i>	<i>Burkholderia ambifaria</i> , <i>B. cepacia</i> , <i>Serratia marcescens</i>	Cucumber seed	22	0	0	0	61
<i>R. solani</i>	...	<i>B. ambifaria</i> , <i>S. marcescens</i>	Cucumber seed (bacteria) and compost (<i>T. virens</i>)	3	0	0	0	...
<i>C. orbiculare</i> , <i>Pseudomonas syringae</i> , <i>Erwinia tracheiphila</i>	<i>Bacillus pumilus</i> , <i>B. subtilis</i> , <i>Curtobacterium flaccumfaciens</i>		Cucumber seed	31	1 (likely)	2	1	59
<i>Fusarium oxysporum</i> , <i>R. solani</i>	<i>B. subtilis</i> , <i>B. amyloliquefaciens</i>	<i>B. licheniformis</i> , <i>Pseudomonas fluorescens</i> , <i>Chryseobacterium balustinum</i>	Tomato seed	3	Unlikely	1	0	15
	...	...	Pepper seed	3	0	0	0	...
<i>Pythium</i> spp.	Two <i>Stenotrophomonas maltophilia</i> strains	<i>P. fluorescens</i>	Sugar beet seed	4	Unlikely	1	0	19
<i>F. oxysporum</i>	Four bacterial strains		Tomato seed	4	0	0	0	68
	...	...	Roots (glasshouse)	4	0	0	0	...
	...	...	Roots (field)	8	0	0	1	...
(continued on next page)								

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^a Total number of treatments with combined use of BCAs, number that resulted in greater control efficacies than predicted based on the Bliss assumption (>Pred), number that resulted in greater control efficacies than the more efficacious component BCA (>Best), and number that were less efficacious than the more efficacious component BCA (<Best). NA = not available.

^b Reference.

TABLE 1. (continued from previous page)

Controlled organism	BCA1	BCA2	Host type	Number of treatments ^a				Ref ^b
				Total	>Pred	>Best	<Best	
<i>Rosellinia necatrix</i>	Four <i>T. atroviride</i> strains		Avocado seed and plants	11	0	0	7	62
<i>Pyricularia oryzae</i>	<i>Pseudomonas fluorescens</i>	<i>Chryseobacterium balustinum</i>	Rice seed and leaves	1	0	0	0	53
<i>Phytophthora infestans</i>	<i>B. cereus</i> , <i>Candida</i> sp., <i>Aspergillus</i> sp., <i>Cellulomonas flavigena</i> , and <i>Cryptococcus</i> sp.		Tomato seed or leaves	5	0	0	0	52
<i>Rhizoctonia solani</i>	<i>B. subtilis</i>	<i>Burkholderia cepacia</i>	Soil in pot (tomato)	3	Unlikely	1	0	69
<i>F. oxysporum</i>	<i>Pseudomonas</i> spp.	<i>F. oxysporum</i> , <i>Acremonium rutilum</i> , <i>Verticillium lecanii</i>	Compost (radish)	22	3 (likely)	6	0	47
<i>P. capsici</i>	<i>Serratia plymuthica</i> , <i>Chromobacterium</i> sp., <i>Lysobacter enzymogenes</i>		Pot pepper plants (media)	3	0	0	0	44
<i>P. capsici</i> , <i>R. solani</i> , <i>F. oxysporum</i> , <i>F. solani</i>	...	...	...	3	Unlikely	3	0	...
<i>F. oxysporum</i>	Two <i>Pseudomonas putida</i> strains		Radish (media)	5	Unlikely	1	0	12
<i>F. oxysporum</i>	<i>T. hamatum</i> , <i>T. harzianum</i> , <i>Paecilomyces lilacinus</i>		Cotton (media)–glasshouse	16	Unlikely	3	8	1
<i>P. debaryanum</i>	...	...	...	16	...	2	12	...
<i>F. oxysporum</i>	<i>Pseudomonas putida</i> , <i>P. fluorescens</i>		Radish (media)–glasshouse	13	Unlikely	3	1	13
<i>Phytophthora capsici</i>	<i>Burkholderia cepacia</i>	<i>T. harzianum</i>	Pepper (media)–field	1	0	0	0	26
<i>Xanthomonas campestris</i>	Five bacterial strains		Wounded anthurium leaves	9	0	0	0	28
<i>Chalara paradoxa</i>	<i>Pichia guilliermondii</i> , <i>Rhodotorula</i> sp., <i>Cryptococcus</i> sp.		Pineapple cut peduncle	1	0	0	0	60
<i>Phytophthora infestans</i>	<i>Pseudomonas</i> spp., <i>Peoria</i> sp., <i>Enterobacter</i> spp., <i>Pantoea agglomerans</i>		Potato tubers	2	0	0	0	65
<i>Gibberella pulicaris</i>	<i>Pseudomonas</i> spp., <i>Peoria</i> spp., <i>Enterobacter</i> spp.		Wounded potato	90	Only tested against the average; individual performance not given but only two treatments are likely better than the better component			63
<i>Phytophthora ramorum</i>	<i>Bacillus subtilis</i> , <i>Bacillus</i> spp., <i>Streptomyces lydicus</i>		Detached leaves (<i>Rhododendron</i> and <i>Camellia</i>)	2	Tested against average: 1 poorer performance and the others similar			25
	<i>T. atroviride</i>	<i>S. lydicus</i>	...	2	...	...	...	...
	<i>T. atroviride</i>	<i>T. virens</i>	...	2	0	0	2	...
<i>Botrytis cinerea</i>	<i>Pichia guilliermondii</i>	<i>Bacillus mycoides</i>	Detached strawberry leaves	4	0	0	0	34
	...	...	...	30	Unlikely	5 (likely)	1 (likely)	...
	...	...	Strawberry roots	2	Unlikely	1	0	...
<i>Botrytis cinerea</i>	<i>T. harzianum</i> , <i>T. atroviride</i> , <i>T. longibrachitum</i>	...	Strawberry fruit (repeated)–field	2	0	0	1	27
<i>Colletotrichum acutatum</i>	...	...	...	5	Unlikely	2	1	...
<i>Botrytis cinerea</i>	<i>Bacillus mycoides</i>	<i>P. guilliermondii</i>	Strawberry (leaflets)–in vitro	8	Unlikely	6 (likely)	0	33
<i>Botrytis cinerea</i>	<i>Bacillus subtilis</i> , <i>T. harzianum</i> , <i>T. atroviride</i>		Strawberry (plants)	36	0	1	2	74
<i>Botryodiplodia theobromae</i>	<i>T. koningii</i> , <i>T. hamatum</i> , <i>Pseudomonas fluorescens</i> , <i>P. putida</i> , <i>Tilletiopsis minor</i> , <i>T. washingtonensis</i>		<i>Annona</i> (repeated)–field	7 (branch)	Unlikely	7	0	35
	...	...	...	7 (flower)	...	6	0	...

epidemiological models have been developed and applied mainly to root pathogens such as *Rhizoctonia solani* (30) but these models do not explicitly deal with the mechanisms of biological control. Nearly all modeling investigations have, thus far, focused on the effect of a single BCA on disease development, mostly for soilborne pathogens (31,32).

Recently, we have developed a generic modeling framework to understand the dynamics of foliar pathogen and BCA populations (40) to predict the likelihood of successful biocontrol in relation to the mechanisms involved. In this generic model, a standard susceptible-infected-removed (SIR) model for host–pathogen dynamics was coupled with one for the pathogen–BCA dynamics via a specific biocontrol mechanism but without considering spatial heterogeneity. Four biocontrol mechanisms (competition, antibiosis, mycoparasitism, and induced resistance) were included within the model framework through effects on the model parameters. This generic mathematical model was then used in a numerical study to understand the dynamics of foliar pathogens in relation to multiple biocontrol mechanisms in a single agent (75). This numerical study showed that biocontrol efficacy was greater for a single BCA with multiple biocontrol mechanisms than individual BCAs with a single mechanism; this difference was greatest for a BCA combining competition with mycoparasitism or antibiosis.

Further numerical simulation was conducted to investigate whether the biocontrol efficacy of combined use of two BCAs with different biocontrol mechanisms is greater than that of a single BCA with either or both of the two biocontrol mechanisms (73). It showed that application of a single BCA with two different mechanisms will always lead to higher levels of disease suppression than the combined use of two BCAs, each with a single biocontrol mechanism. Furthermore, the outcome of the combined use of two BCAs, each with a single but different mechanism, does not necessarily result in appreciable improvement in biocontrol efficacy over an individual BCA with a single mechanism applied alone. Combined use of two BCAs may increase or reduce biocontrol efficacy depending on the type and extent of interactions between them. In most cases, combinational use of BCAs did not lead to an appreciable increase in biocontrol over the more efficacious component and, therefore, was even less likely over the expected efficacy under the assumption of Bliss independence. It is noteworthy that our comparison can be made dynamically through the numerical approach taken. Biocontrol efficacy varied greatly with time, particularly for BCAs with mycoparasitism and, to a lesser extent, antibiosis as their main mode of action. Thus, combined use of BCAs cannot be generally recommended unless their main biocontrol mechanisms and the nature (and magnitude) of their interactions are known.

SUMMARY OF EXPERIMENTAL STUDIES

Selection and presentation criteria. We have gone through all the experimental studies that we could find where a combined use of BCAs was reported. Published studies were selected on the basis of the following criteria: (i) all individual components were identified and tested as well as their mixtures, (ii) tests were done on plant tissues regardless of whether this was *in vitro* or *in vivo*, (iii) summary statistics were available such that the significance of treatment differences could be judged, and (iv) all individual BCAs were used to control the same disease or diseases. Biocontrol mechanisms were often not well understood in these studies.

In reviewing and summarizing published results, we have followed several principles. First, in cases where more than one pathogen- or disease-related variable was recorded, only the actual disease severity or incidence was used to compare treat-

ments. In cases where two BCA components were explicitly described, the two components were then listed as BCA1 or BCA2 in the summary table (Table 1). However, in other cases, this separation was not possible without listing all possible pairs, and all mixture components were then given in a single column. Similarly, in several studies where more than two BCAs were used in mixtures, they were also listed in a single column. Thus, sometimes it was not possible to distinguish two-component treatments from other multiple-component treatments in Table 1.

The number of treatments given in Table 1 may differ from those reported in the original publications. When BCAs were applied in conjunction with other treatments, the total number of BCA mixture treatments was calculated as the product between the number of BCA mixtures and the number of other treatments. For example, if two BCA mixtures were tested under two temperatures, then there would be four mixture treatments. Similarly, when the same experiments were conducted at different sites or times but data were not jointly analyzed or the relative effects varied greatly among experiments, they were then treated as separated studies.

In many studies, explicit comparisons between combined and individual use of BCAs were not either statistically tested or presented. To test the differences, we calculated the expected efficacy for the combined use of BCAs on the basis of Bliss independence because dose-response relationships were not available. Based on the given statistics (e.g., mean and standard error), we then made a judgment on the statistical significance (at the level of 5%) of these comparisons whenever possible. When a clear judgment on the significance could not be made, in some cases we also provided the number of possible significant comparisons. “Not available” was given if no judgment could be made. Our judgment on these comparisons was only made on whether synergistic effects (but not antagonistic interactions) were present. This is because, in most cases, antagonistic interactions among BCAs can be inferred from the comparisons between combined and individual use of BCAs, which can be made more accurately from presented data than in the case of synergistic testing.

Summary of the results. A total of 465 individual treatments from 36 publications involved combined use of BCAs. Most of these dealt with combinational use of two agents although a considerable proportion of these treatments involved more than two agents. Given the data set, it was not possible to draw any conclusions regarding the presence or absence of synergistic or antagonistic interactions in relation to the type of pathogens controlled, BCAs, or host tissues. Rather, we only want to illustrate the overall frequency where such interactions were detected.

Of the 465 treatments, only 30 treatments were from experimental studies of biocontrol in field-grown crops; 49 were cases of wounded apple in storage experiments, 90 were cases of wounded potato, and 48 were cases of detached leaves under *in vitro* conditions. The remaining cases mainly comprised tests in growth chambers and glasshouse compartments, most of which dealt with biocontrol on plants grown from either treated seed or treated growth media in pots for controlling soilborne pathogens.

Only in three cases (7,34,74) was the expected disease control efficacy calculated based on the assumption of Bliss independence. In one study, which examined biocontrol of *Botrytis cinerea* on strawberry in growth chambers, none of the 36 combined BCA treatments resulted in synergistic interactions; however, antagonistic interactions were indicated in 25 treatments (74). In another lab study of *B. cinerea* on strawberry (34), no synergistic interactions among BCAs were observed. Although the expected efficacy was calculated in the other biocontrol study of fungal rotting of apple, statistical testing of synergistic or antagonistic interactions was not presented (7). In most other cases, combined

treatments were only compared with the more efficacious component treatment.

Only in 10 of 465 treatments ($\approx 2\%$) was there statistical evidence for synergistic interactions among component BCAs. In 70 cases, combined use of BCAs resulted in improved efficacy over the more efficacious component. On the other hand, in 41 cases, combined use of BCAs resulted in reduced efficacies compared with the more efficacious component. As we stated before, it is not possible to have an accurate estimate of the number of cases where significant antagonistic interactions were detected, because appropriate statistical comparisons were not carried out in most cases. However, there were at least 64 cases where antagonistic interactions were shown to be significant. If a true test of antagonistic interactions was carried out, a far higher number of treatments would be expected to show antagonistic interactions. For example, in one single study where this test was carried out (74), 25 of the 33 treatments that were not significantly different from the more efficacious component had a significantly lower efficacy than expected under the Bliss independence assumption. If we assume that this ratio also applies to all other studies (of course, this assumption is questionable but this is the best we can do given the presently published information), then we would expect another 268 treatments to have reduced efficacies. Thus, $\approx 66\%$ of the cases would indicate the presence of antagonistic interactions among BCAs.

In summary, both theoretical modeling and experimental studies suggested that, on the basis of the Bliss independence, antagonistic interactions among BCAs were more likely to occur than synergistic effects. Moreover, the proportion of antagonistic interactions may be even higher than estimated here because negative results tend to be under-reported (50). In most cases, the biocontrol efficacy achieved with combined use of two agents is not expected to be greater than that achieved by the more efficacious component. Results from pest biocontrol also suggested that, in contrast to biocontrol of weeds, the observed biocontrol effect resulting from more than one control agent was predominantly due to a single agent in the mixture (14).

CONCLUDING REMARKS

Clear formulation and statistical testing of hypotheses are essential for assessing synergistic or antagonistic effects among BCAs. However, this has rarely been carried out in published studies on combined use of BCAs for control of plant pathogens. It should be stressed that, with additive effects, the efficacy of combined use of BCAs is expected to be always greater than individual components. We should strive for clear formulation and testing of hypotheses in future studies.

Two alternative formulations for hypotheses of independence may be possible. For biocontrol of plant pathogens, Bliss independence might be more appropriate than Loewe additivity because it is more likely that two BCAs act through independent mechanisms. Further research is needed to compare these two methods for assessing biocontrol of plant pathogens, particularly in relation to biocontrol mechanisms. In addition, modeling studies suggested that efficacy from combined use of BCAs depends on the particular biocontrol mechanisms involved. Therefore, to evaluate the potential for improved performance in combined use of BCAs, we need to know the predominant biocontrol mechanisms in each BCA component.

Both experimental and modeling studies suggested that a single BCA with two biocontrol mechanisms usually results in better control than the use of individual or combinations of two BCAs with single mechanisms of action. Thus, future selection of BCAs should place emphasis more on potential microorganisms possessing multiple biocontrol mechanisms.

Unlike drug interactions, BCAs are living organisms and, thus, biocontrol efficacy is expected to vary with time, as shown in numerical modeling investigations (40,75). Thus, the presence of synergistic or antagonistic interactions and their magnitude thus vary with time. Current biocontrol studies often assess disease development at a single time point, thus ignoring the temporal dynamics in biocontrol of plant pathogens. Future studies need to consider this aspect to fully understand biocontrol outcomes.

One of the potential advantages in the combined use of BCAs is that it may lead to reduced variability in biocontrol efficacies (33). This increased stability is also often observed in cultivar mixtures (66). Given the general lack of synergistic effects, future research on combined use of BCAs may need to determine whether this increased stability in biocontrol is a general phenomenon or specific to certain systems, and whether it is dependent on biocontrol mechanisms.

It is likely that biocontrol efficacy via augmentative treatment with BCAs under natural conditions is even more variable than those reported in controlled studies, as often observed in field trials. Where naturally occurring biological control is manifested, such as in suppressive soils, the biocontrol activity is likely to be via a combination of many agents (and not exclusively biological) that has developed over considerable time and has been selected through prevailing conditions. It is also noteworthy that a distinction can be made between general and specific suppressiveness (71), with the former acting in the bulk soil and the latter generally in the rhizosphere. Future studies are needed to understand whether biocontrol efficacy and its variability are associated with the microbial community structure where the biocontrol action takes place.

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