

Combinations of Biocontrol Agents for Management of Plant-Parasitic Nematodes and Soilborne Plant-Pathogenic Fungi¹

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Abstract: Numerous microbes are antagonistic to plant-parasitic nematodes and soilborne plant-pathogenic fungi, but few of these organisms are commercially available for management of these pathogens. Inconsistent performance of applied biocontrol agents has proven to be a primary obstacle to the development of successful commercial products. One of the strategies for overcoming inconsistent performance is to combine the disease-suppressive activity of two (or more) beneficial microbes in a biocontrol preparation. Such combinations have potential for more extensive colonization of the rhizosphere, more consistent expression of beneficial traits under a broad range of soil conditions, and antagonism to a larger number of plant pests or pathogens than strains applied individually. Conversely, microbes applied in combination also may have antagonistic interactions with each other. Increased, decreased, and unaltered suppression of the target pathogen or pest has been observed when biocontrol microbes have been applied in combination. Unfortunately, the ecological basis for increased or decreased suppression has not been determined in many cases and needs further consideration. The complexity of interactions involved in the application of multiple organisms for biological control has slowed progress toward development of successful formulations. However, this approach has potential for overcoming some of the efficacy problems that occur with application of individual biocontrol agents.

Key words: bacteria, biocontrol, combination, fungi, microbe, nematode.

Numerous microbes are antagonistic to plant-parasitic nematodes and plant-pathogenic fungi, and some of these organisms reduce pathogen populations and/or disease (Adams and Ayers, 1982; Alabouvette et al., 1993; Chen and Dickson, 1998; Harman, 1991; Kerry, 1998; King and Parke, 1993; Lumsden and Locke, 1989; Marois et al., 1982; Nelson, 1988; Rodríguez-Kábana and Morgan-Jones, 1988; Sayre, 1986; Siddiqui and Mahmood, 1996a, 1999; Sikora and Hoffmann-Hergarten, 1993; Stirling, 1991; Weller and Cook, 1983). However, biocontrol microbes often are not thought of as acceptable alternatives for pesticides. Reasons for this include lack of broad spectrum activity, inconsistent performance, and slower (and sometimes less complete) action by the biocontrol agents when compared with pesticides. Additionally, only a few of these organisms have been developed into commercial biocontrol products available to the grower. This is because the leap from recognition of a potentially useful biocontrol agent to mass culture, formulation, wide-scale testing, shelf-life improvement, registration, marketing, and delivery has evidently proved so great that research has not resulted in many commercial successes

for management of plant-pathogenic fungi and nematodes. Commercially available biocontrol products for soilborne plant-pathogenic fungi include microbes such as *Bacillus*, *Burkholderia*, *Coniothyrium*, *Fusarium*, *Gliocladium*, *Pythium*, *Streptomyces*, *Talaromyces*, and *Trichoderma* (Fravel, 2000). For nematode management, even fewer commercial formulations are available; examples of products registered for biocontrol include formulations containing *Burkholderia* and *Paccilomyces*.

Of the problems listed above, inconsistent field performance often restricts commercial development of biocontrol agents. This inconsistency can be caused by a large number of biotic and abiotic factors. Biotic factors include interactions with nontarget organisms, damage caused by nontarget pathogens and pests, degree of rhizosphere and/or soil colonization by a biocontrol agent, initial population levels of the target organism, susceptibility of the host plant to the target pathogen or pest, host plant species, and host plant cultivar (Duffy et al., 1996; Hebbbar et al., 1998; Kerry, 1998; Kerry and Bourne, 1996; Pierson and Weller, 1994; Sikora and Hoffmann-Hergarten, 1993; Stirling, 1991). Abiotic factors affecting efficacy of biocontrol agents include climate, and physical and chemical composition of the rhizosphere (Ownley et al., 1992; Sikora and Hoffmann-Hergarten, 1993; Stirling, 1991). With so many factors affecting the activity of beneficial microbes, it is not surprising that an individual biocontrol agent is differentially active in various soil environments.

One approach to overcoming inconsistent performance under varying environmental conditions is to include two or more biocontrol agents in a preparation.

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Potential advantages of biocontrol agents applied in combination include: (i) multiple modes of action against the target pathogen or nematode; (ii) ability to affect more than one stage of the life cycle of the target organism; (iii) activity of microbes during different times in the growing season; (iv) increased consistency in performance over a wider range of soil conditions, stemming from the different environmental niches of the applied microbes; and (v) potential to select organisms that affect more than one plant pathogen or pest, thus increasing the spectrum of uses for the product (Crump, 1998; Larkin et al., 1998; Lemanceau and Alabouvette, 1991; Lemanceau et al., 1992; Pierson and Weller, 1994; Siddiqui and Mahmood, 1996a).

This paper considers studies that have been done with living microbes (bacteria and fungi) that are applied in combination to the spermosphere or rhizosphere and specifically designated as biocontrol agents. The discussion does not include amendments, products designed to increase activity of organisms already present in the soil, living agents not specifically registered for biocontrol, root-nodulating bacteria, or natural products derived from microbes.

Examples of combinations of biocontrol agents for management of plant parasitic nematodes: A number of research investigations indicate that biocontrol combinations may have a future for management of plant-parasitic nematodes. In these studies, certain microbe combinations resulted in increased plant vigor or yield, and (or) reduction of nematode populations or penetration on roots, compared with individual applications of the biocontrol agents (de Leij et al., 1992; Duponnois et al., 1998; Gautam et al., 1995; Hojat Jalali et al., 1998; Khan et al., 1997; Maheswari and Mani, 1988; Perveen et al., 1998; Siddiqui et al., 1999a, 1999b; Siddiqui and Husain, 1991; Siddiqui and Mahmood, 1993, 1995a, 1995b, 1996b; Sosamma and Koshy, 1997; Vidya and Reddy, 1998; Youssef and Ali, 1998; Zaki and Maqbool, 1992). The effects on nematode populations included suppression in numbers of females, eggs, egg masses, juveniles, or galls.

Root-knot nematodes (*Meloidogyne* spp.) have been the subject of many of the studies on biocontrol combinations. This research has resulted in the identification of a number of successful biocontrol combinations that act against nematodes in this genus. In one study, the bacterium *Bacillus subtilis* and the fungus *Paecilomyces lilacinus* were tested for suppression of *Meloidogyne incognita* on tomato in pots containing steamed soil (Gautam et al., 1995). Alone or combined, the microbes increased plant height and weight and suppressed numbers of root galls, females, eggs, and second-stage juveniles (J2). However, the combination of these two biocontrol agents suppressed nematode populations beyond application of agents individually. There was some increase of plant height and weight over individual applications as well, although the com-

bination did not have a substantial overall effect on plant vigor compared to *P. lilacinus* alone. In another investigation, tomato seedlings were planted into pots containing an unsterilized peat/sand/compost mix. The biocontrol agents tested were the fungus *Verticillium chlamydosporium* and the bacterium *Pasteuria penetrans*. Individual application of these microbes generally reduced root galling. However, a combination of the two organisms caused the largest reduction in galling. Number of female nematodes was not affected by any biocontrol treatment, but individual and combined agents decreased numbers of eggs and J2, with an enhanced effect by the combination on suppression of nematode populations after longer growing periods (de Leij et al., 1992). The nematode-trapping fungus *Arthrobotrys oligospora* was tested with each of 11 strains of bacteria—*Pseudomonas mendocina*, *Enterobacter cloacae*, *Bacillus licheniformis*, and several unidentified strains—for effects on *Meloidogyne mayaguensis* on tomato in autoclaved soil. Numbers of J2 were suppressed with three of the combinations, all of which included *A. oligospora* and different unidentified bacteria (Duponnois et al., 1998). These same combinations did not result in the highest plant biomass.

Combinations of biocontrol agents have not been tested as frequently against cyst nematodes (*Heterodera* spp.). However, studies demonstrating enhanced efficacy with combinations have been reported. For example, three fungi (*Embellisia chlamydospora*, *V. chlamydosporium*, and a sterile fungus) were tested for effects on *Heterodera schachtii* on sugar beet in autoclaved soil (Hojat Jalali et al., 1998). In this growth-chamber experiment, none of the fungi applied individually suppressed the number of females or cysts in pots, nor did the *Embellisia-Verticillium* combination. However, combinations of either *Embellisia* or *Verticillium* with the sterile fungus significantly suppressed numbers of females and cysts.

Studies have been conducted in which no enhanced benefits were observed with combinations of biocontrol agents. For example, the fungi *Hirsutella rhossiliensis* and *V. chlamydosporium*, applied against *Meloidogyne hapla* on lettuce, were effective in reducing J2 numbers in seedlings when applied alone or in combination (Viaene and Abawi, 2000). However, combining the two fungi did not enhance activity against *M. hapla* when compared with individual application of these biocontrol agents. The individual fungi and their combination did not affect lettuce weight, nematode egg production, or root galling at the highest nematode inoculum level. Zaki and Maqbool (1991) found that while the biocontrol agents *P. penetrans*, *P. lilacinus*, *Talaromyces flavus*, and *B. subtilis* generally reduced root-knot nematode indices, combinations were not more effective than individual application of these biocontrol agents.

While some microbe combinations enhance or have no significant impact on biological control, other com-

binations result in decreased biocontrol. The fungus *Trichoderma virens* and bacteria from the *Burkholderia cepacia* complex are sold commercially as the biocontrol products Soilgard (ThermoTrilogy Corp., Columbia, MD) and Deny (Stine Microbial Products, Madison, WI), respectively. Strains of these organisms were found to produce extracellular factors in vitro that decreased *M. incognita* egg hatch and J2 mobility (Meyer et al., 2000). In greenhouse studies, individual application of the microbes as seed coats followed by root drenches suppressed root-knot nematode populations (*M. incognita*) on bell pepper compared with untreated plants. In contrast, combinations of *T. virens* with the bacteria were not as effective. Nematode populations (eggs + J2 per g of root) on plants treated with *B. cepacia*-*T. virens* combinations were not significantly different from those on the control plants (Meyer et al., 2001). Incompatibility among biocontrol organisms has been observed in other studies. *Bacillus thuringiensis*, *Paecilomyces marquandii*, *Streptomyces costaricanus*, and a *B. thuringiensis*/*S. costaricanus* combination were applied as suspensions to soil and studied for effects on *M. hapla* on lettuce (Chen et al., 2000). Each of the biocontrol agents, applied individually, reduced egg numbers in both methyl bromide-fumigated and nonfumigated microplots compared to untreated controls, and reduced root galling in nonfumigated soil. However, the combination did not suppress root galling or egg numbers. Individually applied microbes increased lettuce head weight in nonfumigated soil compared to untreated controls, whereas the combination did not. The combination therefore was not as effective as individual treatments for increasing lettuce head weight or for decreasing nematode populations. In a study with potted banana plants, *S. costaricanus*, *B. thuringiensis*, and *P. marquandii* in combinations were generally not as efficacious for nematode management as the organisms applied individually against *Radopholus similis* and *Helicotylenchus multicinctus* (Esnard et al., 1998).

Examples of combinations of biocontrol agents for management of soilborne plant-pathogenic fungi. Combinations of biocontrol agents have been tested for suppression of soilborne fungal pathogens. As with the use of biocontrol agents for nematode management, some combinations improved suppression of soilborne plant-pathogenic fungi (Duffy et al., 1996; Duffy and Weller, 1995; Leeman et al., 1996; Lemanceau and Alabouvette, 1991; Lemanceau et al., 1992; Park et al., 1988; Pierson and Weller, 1994), while some combinations did not provide any advantage (Dandurand and Knudsen, 1993; Hubbard et al., 1983; Minuto et al., 1995). For example, cucumber seed treatments containing combinations of two biocontrol bacteria provided significantly greater suppression of cucumber seedling pathogens in a field soil naturally infested with *Pythium* and *Fusarium* spp. than was obtained with the individual bacterial strains (Roberts et al., 1997).

Impressive results have come from greenhouse trials performed by Mao et al. (1998) evaluating the combined application of *T. virens* G1-3 and *B. cepacia* Bc-F for the control of seed and seedling diseases of tomato caused by the plant-pathogenic fungi *Rhizoctonia solani* and *Pythium ultimum*, alone or in combination with the plant-pathogenic fungi *Sclerotium rolfsii* and *Fusarium oxysporum* f. sp. *lycopersici*. Greenhouse trials were also performed with G1-3 and Bc-F for the control of *R. solani*, *P. ultimum*, and *S. rolfsii* alone or in combination with *Phytophthora capsici* on pepper. Tomato seeds treated with G1-3 and Bc-F, individually and in combination, and sown in soil-less mix infested with the above pathogens resulted in seedling stands comparable to that of the noninfested controls. However, on pepper, only seed treatments with the combination of G1-3 and Bc-F resulted in stands similar to the noninfested controls. Surviving tomato and pepper plants were transplanted into field plots infested with a variety of pathogens. At plant maturity, the combined G1-3 plus Bc-F application resulted in values for fresh weight and disease severity on pepper, and fruit yield for tomato, that were significantly better than individual applications of these biocontrol agents or the pathogen check (Mao et al., 1998). In this case, preparations containing G1-3 combined with Bc-F generally provided more effective disease suppression than preparations containing the individual applications.

In other studies, corn seeds treated with *T. virens* G1-3, *B. cepacia* Bc-B, *B. cepacia* Bc-1, or G1-3 in combination with Bc-B or Bc-1, were evaluated in a nonsterile field soil artificially infested with several species of *Pythium* and *Fusarium*. G1-3 in combination with *B. cepacia* Bc-B provided significantly greater biocontrol than all other seed treatments (Mao et al., 1996). In field trials, only the combined application of G1-3 with Bc-B provided significant disease control on corn in soil artificially infested with several different species of *Pythium* and *Fusarium*. The combination of *T. virens* G1-21 with the bacterium *B. cepacia* Bc-F provided significantly greater control of *R. solani* on cucumber than G1-21 or Bc-F applied individually under very high levels of disease pressure (Roberts and Lewis, unpubl.). Interestingly, combinations of G1-21 with a second strain of *B. cepacia* did not increase biocontrol activity. Furthermore, combinations of G1-21 with other bacteria, or preparations consisting only of combinations of bacteria, were reduced in efficacy compared with the biocontrol agents applied alone (Roberts and Lewis, unpubl.).

Examples of combinations of biocontrol agents for management of plant-parasitic nematodes and soilborne plant-pathogenic fungi: Suppression of more than one plant pest or pathogen would increase the value of an applied biocontrol agent. As a consequence, some microbe combinations have been tested for activity against plant-pathogenic nematodes and fungi (Khan et al., 1997;

Perveen et al., 1998; Siddiqui et al., 1999a, 1999b; Siddiqui and Mahmood, 1993, 1995a, 1995b, 1996b). These studies often report enhanced activity with combined microbes. For example, the beneficial fungi *P. lilacinus* and *Trichoderma harzianum* were applied against *M. incognita* and *Fusarium solani* on potted papaya in steamed soil (Khan et al., 1997). Each biocontrol agent applied alone was able to improve plant vigor, reduce nematode numbers, and decrease incidence of root rot. However, the combined application was even more effective. In another study, pigeonpea was planted in autoclaved soil and treated with the fungi *T. harzianum* (TH), *V. chlamydosporium* (VC), and *Glomus mosseae* (GM) (Siddiqui and Mahmood, 1996b). Tested combinations were TH+GM, VC+GM, VC+TH, and VC+TH+GM. The combinations enhanced activity against the *Heterodera cajani*-*Fusarium udum* wilt disease complex, compared with applications of individual biocontrol agents. Plant height and shoot dry weights were increased (compared to application of individual agents), numbers of females and cysts per root system were decreased by all but VC+GM, J2 populations were reduced, and wilting indices decreased.

Ecological considerations: While some strain combinations are beneficial for biocontrol, clearly not all combinations of strains result in significantly improved and consistent disease suppression. Several authors have suggested that for improved biocontrol performance to occur, strains combined in preparations must be compatible (Baker, 1990; Janisiewicz, 1996; Janisiewicz and Bors, 1995; Raaijmakers et al., 1995). More consideration must be given to interactions among strains combined in biocontrol preparations.

Almost all biocontrol interactions leading to disease suppression can be placed into one or more of the following general categories of mechanisms: antibiosis, induced resistance of plants, competition for resources such as nutrients, and predation/parasitism (Kerry, 1998; Larkin et al., 1998; Siddiqui and Mahmood, 1999; Sikora and Hoffmann-Hergarten, 1993). The positive effects resulting from the combination of some biocontrol agents, leading to more effective disease suppression, are likely the result of additive or synergistic effects of the combined mechanisms of disease suppression against the pathogen. The negative interactions resulting in a reduction in biocontrol efficacy are likely the result of these mechanisms of suppression being directed at the companion biocontrol agent within the preparation, in addition to being directed at the plant pathogen.

Considerable potential exists for antagonism between the microbes combined in biocontrol preparations that may lead to decreased management performance. Microbes combined in biocontrol preparations are potentially antagonistic with each other through parasitism and antibiosis. For example, nematophagous fungi can exhibit predacious activity toward other fungi

as well as toward nematodes (Rosenheim et al., 1995). Antibiotics such as phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol, hydrogen cyanide, and other metabolites contribute to the activity of many biocontrol agents (Barker and Koenning, 1998; Keel et al., 1992; Pierson and Thomashow, 1992; Sikora and Hoffmann-Hergarten, 1993; Thomashow and Weller, 1988; Vincent et al., 1991; Weller and Thomashow, 1993) but also may be inhibitory to other biocontrol agents (Pierson and Weller, 1994). Evidence that antibiotics are produced in the rhizosphere and are inhibitory to specific soil microbes comes from genetic studies with antibiotic-producing biocontrol bacteria (Keel et al., 1992; Thomashow and Weller, 1988; Vincent et al., 1991). For example, *Pseudomonas fluorescens* RS111 was strongly inhibited in vitro by *Pseudomonas putida* RE8; strain RE8 was not inhibited by strain RS111. This inhibition was due to a diffusible compound, possibly an antibiotic, that was released into the agar medium. Strain RS111a, a spontaneous mutant of strain RS111, was less sensitive to inhibition by RE8 than RS111 in vitro. Preparations containing incompatible strains (RE8 and RS111) and compatible RE8 and RS111a strains were used for control of *Fusarium* wilt of radish. The incompatible strain combination provided significant disease suppression. However, this suppression was similar to that obtained with treatments containing strain RE8 or strain RS111 applied individually. In contrast, the compatible strain combination gave significantly greater disease suppression than the pathogen check, treatments containing the strains applied individually, and the treatment containing the incompatible strain combination (de Boer et al., 1997).

Antagonism between biocontrol agents within a particular biocontrol preparation may also arise due to competition for limiting resources, such as nutrients, in the rhizosphere. Competition has been defined as the active demand in excess of the immediate supply of material on the part of two or more organisms (Clark, 1965). The result is restricted population size or microbial activity of one or more of the competitors (Paulitz, 1990). Several levels of competition for nutritional resources may be at work in the rhizosphere when multiple biocontrol strains are added (e.g., competition between the biocontrol strains or competition between biocontrol strains and the indigenous microflora and fauna), which may include plant pathogens and pests (Paulitz, 1990). Evidence that the rhizosphere environment is nutrient-limiting comes from the biocontrol literature (Paulitz, 1990), where various approaches have demonstrated that the rhizosphere can be limiting in reduced carbon, nitrogen, or iron (Chen et al., 1988; Kloepper et al., 1980; Loper, 1988; Weller et al., 1988).

Competition for nutrients among microbes resulting in antagonism was shown by Wilson and Lindow (1994a, 1994b). Their research analyzed the interactions between bacterial biocontrol agents and bacterial

plant pathogens on leaf surfaces. Competition for nutrients between nonice-nucleating and ice-nucleating strains of *Pseudomonas syringae* resulted in antagonism as indicated by reduced colonization by ice-nucleating strains of *P. syringae* (Lindow, 1983, 1985, 1987; Lindow et al., 1983a, 1983b). Wilson and Lindow (1994b) determined that coexistence of epiphytic bacteria was inversely correlated with the similarity in reduced carbon utilization between the interacting strains. Strains with high niche overlap were antagonistic with each other through competition for limiting nutritional resources. Conversely, coexistence of bacterial species on leaf surfaces was mediated through nutritional niche differentiation, the utilization of different nutrients by coexisting strains (Wilson and Lindow, 1994b). The work of Wilson and Lindow suggests that biocontrol agents with low nutritional niche overlap in the rhizosphere should coexist and not impact the performance of other biocontrol agents. However, a predictive model relating nutritional niche overlap between biocontrol agents and biocontrol performance has not been formulated or tested for the rhizosphere.

Coexistence among species within a community has been shown to be mediated through mechanisms other than nutritional niche differentiation, such as temporal or spatial separation of species (Evans et al., 1989; Niemela, 1993). Root and stem interiors represent additional habitats, spatially distinct from the rhizosphere, to be colonized by biocontrol agents. It may be possible to decrease antagonism between biocontrol organisms by applying agents that occupy distinct spatial niches (e.g., the rhizosphere and the internal portions of plants) (Chao et al., 1986). However, there is a paucity of information on the importance of spatial separation of biocontrol agents to biocontrol efficacy. Clearly, work regarding compatibility to inhibitory molecules and nutritional, temporal, and spatial niche overlap between biocontrol agents, and its relation to biocontrol efficacy, is needed.

Conclusions: It is postulated that disease-suppressive soils are the result of the concerted action of many microorganisms (Alabouvette, 1986; Lemanceau and Alabouvette, 1991; Schippers, 1992). Some nematode-suppressive soils may be exceptions, with one or two agents providing the beneficial activity, but application of one microbe does not generally emulate natural suppressiveness (Kerry and Bourne, 1996). Consequently, the application of combinations of disease-suppressive microbes may more closely mimic these natural suppressive soils than application of individual antagonistic microbes, and provide a more viable disease control strategy (Duffy et al., 1996).

Studies with treatments containing combinations of two or more antagonists have shown that strain combinations can be capable of providing effective control of a number of pathogens on multiple crop species. The effectiveness of strain combinations cannot always be

predicted from the individual performance of microbes as biocontrol agents, in part because strains are often combined without consideration of interactions among biocontrol agents. More work needs to be done to minimize the negative interactions while maintaining the positive interactions resulting from the co-application of biocontrol agents. It is hoped that, with a better understanding of the ecological basis of the interactions among microbes applied for biocontrol, levels of performance associated with disease-suppressive soils may be approximated.

From the commercialization perspective, production and quality control difficulties are increased when two or more microbes are involved. Specific formulations for multiple microbes will be necessary, and the shelf lives of more than one organism have to be ensured. Development and production costs are potentially increased, and registration is likely to be more difficult and expensive. Clearly, the acceptance of biocontrol combinations will depend on an increase in efficacy and resulting economic benefit that justify the use of combinations over application of individual microbes. Currently, products containing more than one species of microbe are not sold specifically as biocontrol agents for plant-parasitic nematodes. However, combinations are sold for management of plant-pathogenic fungi. One such product is BINAB TF WP (BINAB Bio-Innovation AB, Karlsborg, Sweden), composed of *T. harzianum* and *Trichoderma polysporum*. Another example is *T. harzianum* with *Trichoderma viride*, used for the products Trichopel, Trichobject, Trichodowels, and Trichoseal (Agrimm Technologies Ltd., Christchurch, New Zealand). Formulations with combined organisms can show improvements over applications of individual microbes. For example, the formulations can be more versatile, have superior activity against the pathogens, or be active over a wider range in temperature and other environmental conditions, e.g., moisture, pH, soil type, etc. (Dodd, pers. comm.; Ricard, pers. comm.).

Formulations containing multiple microbes could be useful for management of nematodes and soilborne plant-pathogenic fungi. Future work in this area should include more microplot and field tests. It is also possible that some successful combinations have been efficacious because the total amount of inoculum was higher (each microbe having been applied at the same rate that was used for individual applications); continued work will determine whether this is the case. Interactions among biocontrol agents should be studied in detail, and knowledge of the ecology of the organisms will help determine whether activity will be complementary. Formulations and delivery systems need to be tailored to the multiple organisms involved. As these and related areas continue to be addressed, the advantages of biocontrol applications containing two or more microbes may be realized.

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