Symposium

The Nature and Application of Biocontrol Microbes: *Bacillus* spp.

Induced Systemic Resistance and Promotion of Plant Growth by *Bacillus* spp.

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ABSTRACT

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Elicitation of induced systemic resistance (ISR) by plant-associated bacteria was initially demonstrated using *Pseudomonas* spp. and other gram-negative bacteria. Several reviews have summarized various aspects of the large volume of literature on *Pseudomonas* spp. as elicitors of ISR. Fewer published accounts of ISR by *Bacillus* spp. are available, and we review this literature for the first time. Published results are summarized showing that specific strains of the species *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* elicit significant reductions in the incidence or severity of various diseases on a diversity of hosts. Elicitation of ISR by these strains has been demonstrated in greenhouse or field trials on tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, *Arabidopsis* sp., cucumber, loblolly pine, and two tropical crops (long cayenne pepper and green kuang futsoi). Protection resulting from ISR elicited by *Bacillus* spp. has been reported against leaf-spotting fungal and bacterial pathogens, systemic viruses, a crown-rotting fungal pathogen, root-knot nematodes, and a stem-blight fungal pathogen as well as damping-off, blue mold, and late blight diseases. Reductions in populations of three insect vectors have also been noted in the field: striped and spotted cucumber beetles that transmit cucurbit wilt disease and the silver leaf whitefly that transmits *Tomato mottle virus*. In most cases, *Bacillus* spp. that elicit ISR also elicit plant growth promotion. Studies on mechanisms indicate that elicitation of ISR by *Bacillus* spp. is associated with ultrastructural changes in plants during pathogen attack and with cytochemical alterations. Investigations into the signal transduction pathways of elicited plants suggest that *Bacillus* spp. activate some of the same pathways as *Pseudomonas* spp. and some additional pathways. For example, ISR elicited by several strains of *Bacillus* spp. is independent of salicylic acid but dependent on jasmonic acid, ethylene, and the regulatory gene *NPR1*—results that are in agreement with the model for ISR elicited by *Pseudomonas* spp. However, in other cases, ISR elicited by *Bacillus* spp. is dependent on salicylic acid and independent of jasmonic acid and *NPR1*. In addition, while ISR by *Pseudomonas* spp. does not lead to accumulation of the defense gene *PR1* in plants, in some cases, ISR by *Bacillus* spp. does. Based on the strains and results summarized in this review, two products for commercial agriculture have been developed, one aimed mainly at plant growth promotion for transplanted vegetables and one, which has received registration from the U.S. Environmental Protection Agency, for disease protection on soybean.

Plant growth-promoting rhizobacteria (PGPR) are among the various groups of plant-associated microorganisms that can elicit plant defenses (28). In concert with the terminology used by van Loon and Glick in their recent review of PGPR (28), we will use the term induced systemic resistance (ISR) for the process whereby treatment of plants with PGPR elicits host defense as indicated by reduction in the severity or incidence of diseases caused by pathogens that are spatially separated from the inducing agent. With each of the cases of ISR elicited by *Bacillus* spp. discussed in this review, spatial separation of the pathogen from the bacteria was confirmed in the cited manuscript or in a previous manuscript with the same bacterial strain.

In the 1990s, several PGPR-based products became commercially available in the United States, and more are currently under development. Most of these products contain strains of *Bacillus* PGPR. Earlier attempts to commercialize products containing fluorescent pseudomonad strains of PGPR generally failed due to lack of long-term viability of these asporogenous bacteria. Although commercialization of PGPR is mainly proceeding with *Bacillus* spp. rather than pseudomonads, the preponderance of research on PGPR as elicitors of growth promotion or ISR employs PGPR strains that are fluorescent pseudomonads. In this review, we summarize research on *Bacillus* PGPR. Our main emphasis is on *Bacillus* spp., which elicit ISR; however, since most such *Bacillus* spp. also promote plant growth, this review summarizes work on both growth promotion and ISR. We concentrate the review on refereed journal articles with some inclusion of previously unpublished work. The results of new work are marked as such in the review.

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Greenhouse reports of systemic disease protection. Workers at Montana State University (1,2) found two strains of *B. pumilus* (strains 203-6 and 203-7) and one of *B. mycoides* (strain Bac J) that reduced the severity of Cercospora leaf spot of sugar beet, caused by *Cercospora beticola* Sacc. Strain Bac J was isolated from sugar beet leaves in the field. For greenhouse tests of ISR, the strain was sprayed onto one leaf of test plants at approximately 1.0 log 8 CFU/ml. The leaf was bagged, and the plants were challenge-inoculated by being sprayed with spore suspensions 3 days after treatment with Bac J (1). Significant reductions in disease severity were obtained by treatment with Bac J on both a highly susceptible and a moderately resistant variety of sugar

beet (1). Strains 203-6 and 203-7, originating from sugar beet embryos, were selected in a subsequent study aimed at developing a rapid selection system for bacteria that elicit ISR against Cercospora leaf spot (2). Both strains significantly reduced disease severity to a level that was not significantly different from the protection afforded by strain Bac J and acibenzolar-*S*-methyl, a chemical elicitor of systemic acquired resistance.

Further evidence that some strains of *Bacillus* sp. can elicit systemic protection against foliar pathogens was provided in a comprehensive study aimed at determining mechanisms for disease protection afforded by the use of composts (15). Work at Ohio State University previously demonstrated that some composts elicit ISR against foliar pathogens (5,6). In an attempt to find rhizobacteria that could account for the observed elicitation of ISR by composts, Krause et al. (15) screened bacteria isolated from two composts for their capacity to elicit systemic protection against *Xanthomonas campestris* pv. *armoraciae*. Eleven rhizobacteria were shown to elicit significant reductions in disease severity in two of three repeated assays, and four of the top-performing strains were shown to be *Bacillus* spp. Krause et al. (15) noted that while previous studies (41,42) showed that about 25% of rhizobacteria isolated from composts could suppress *Pythium* spp., in the current study, <1% of isolates suppressed bacterial leaf spot on radish. Thus, suppression of *Pythium* sp. may result mainly by antibiosis, whereas suppression of bacterial leaf spot resulted from ISR.

Reductions in the severity or incidence of viral diseases have been reported in greenhouse studies with some strains of *Bacillus* spp. Zehnder et al. (35) conducted a greenhouse screen of PGPR for the potential to elicit ISR against *Cucumber mosaic virus* (CMV) on tomato. Each of the three strains selected from the 26 tested significantly reduced the mean percentage of symptomatic plants in each of five experiments, with disease incidence ranging from 88 to 98% in the nonbacterized controls and 32 to 58% for the PGPR-treated plants. Similarly, Murphy et al. (16) used five two-strain combinations of *Bacillus* spp. incorporated into potting mix. When compared with controls, all the bacterial treatments significantly reduced disease severity based on symptoms, decreased disease incidence based on enzyme-linked immunosorbent assay (ELISA), and decreased virus accumulation. Significant increases in plant fresh weight and number of fruits and flowers resulted when treated plants were not challenge-inoculated with CMV. Murphy et al. (16) suggested that this plant growth response resulted in plants that exhibited mature plant resistance, as the level of protection from bacterial-treated plants was generally similar to that observed in nonbacterized plants that were 10 days older. Elicitation of systemic protection against CMV by *B. pumilus* strain SE34 was also noted in tobacco in unpublished work by C.-M. Ryu. The plant response is shown in Figure 1, and the experiment is discussed below in the section on mechanisms.

The severity of blue mold of tobacco, caused by *Peronospora tabacina* Adam, has been reduced by treatment with *Bacillus* spp. (37–39). Zhang et al. (38) developed detached leaf and microtiter plate bioassays to assess elicitation of ISR by bacteria and compared results in these bioassays with results in pot trials in the greenhouse. *B. pasteurii* C-9 and *B. pumilus* SE34 and T4, when applied as soil drenches of three tobacco cultivars, resulted in significant reductions in the mean percentage of leaf area with lesions caused by *Peronospora tabacina*. Sporulation of the pathogen was also significantly decreased by treatment with all three bacterial strains in pot trials. Strains SE34 and T4, but not strain C-9, significantly reduced disease severity in the detached leaf and microtiter plate bioassays. Sporulation of the pathogen was significantly reduced by treatment with all three strains, compared with that of the nonbacterized control, in the detached leaf bioassay. In a separate study (39), the same three bacterial strains were used to explore the relationship between elicitation of plant growth promotion and ISR. When the bacteria were applied as a seed treatment alone, tobacco growth was significantly enhanced by strains SE34 and C-9 but not by T4. ISR was elicited by C-9 but not by SE34 or T4. When the bacteria were applied by seed treatments followed by soil drenches, all strains significantly enhanced plant growth. ISR was elicited by all strains when the time interval between the last application of bacteria and challenge-inoculation with *Peronospora tabacina* was 6 weeks. When this time interval was increased to 8 weeks, strains SE34 and T4, applied in the combination treatment, elicited ISR, whereas strain C-9 did not. Overall, the results indicated an association between the capacity of the tested strains of *Bacillus* spp. to promote growth and elicit ISR.

Greenhouse screening of *Bacillus* spp. that had elicited ISR on other crops was conducted in Thailand (10) as a first step toward employment of bacterial-elicited ISR in tropical agriculture. This study used four different host–pathogen systems: tomato and *Ralstonia solanacearum*, which causes bacterial wilt; long cayenne pepper (*Capsicum annuum* var. *acuminatum*) and *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., which causes anthracnose; green kuang futsoi (*Brassica chinensis* var. *parachinensis*) and *Rhizoctonia solani* Kühn, which causes damping-off; and cucumber and CMV. The goal of the study was to find mixtures of

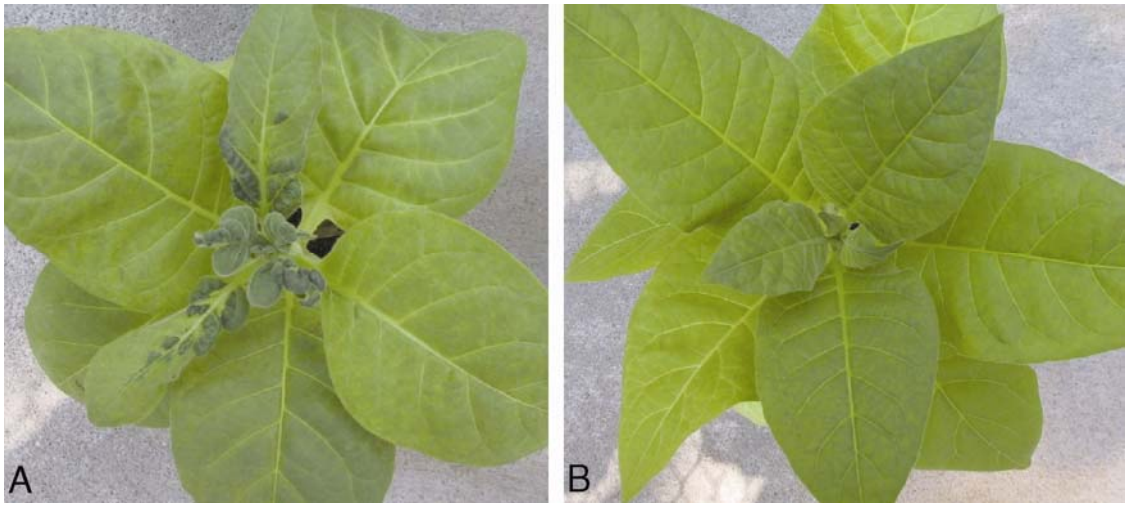

Fig. 1. Systemic protection against *Cucumber mosaic virus* on tobacco elicited by *Bacillus pumilus* strain SE34. **A** shows the nonbacterized, virus-inoculated control. **B** shows protection resulting from treatment of tobacco at the time of transplanting with strain SE34. Photo provided by C.-M. Ryu and has not previously been published.

Bacillus spp. that elicited systemic protection in all four host–pathogen systems. A total of seven individual strains and 11 combinations of two strains were tested and were applied as seed treatments and drenches. One strain significantly reduced incidence or severity of all four diseases. The results are noteworthy for two reasons. First, they indicate that ISR elicited by specific *Bacillus* PGPR strains can protect hosts under tropical conditions. Second, the results show that mixtures of two bacterial strains were superior to individual strains for eliciting significant protection in multiple hosts against different pathogens.

Cucurbit wilt disease is a vascular disease common in the southeastern United States on cucumber and is caused by *Erwinia tracheiphila*. Zehnder et al. (33) conducted greenhouse tests to determine how treatment of cucumber with *B. pumilus* strain INR7 affected disease severity. Bacteria were applied both as seed treatments and drench. In this study, one of the insect vectors of the disease was used to transmit the pathogen from diseased to noninfected plants with or without bacterial treatment. On plants that had been treated with INR7, the number of wilted leaves per plant was significantly less than on plants without bacterial treatment. Elicitation of systemic protection against cucurbit wilt was also noted in field trials as discussed in the section on field trials.

Systemic protection of tomato against late blight, caused by *Phytophthora infestans* (Mont.) de Bary, was demonstrated with *B. pumilus* strain SE34 incorporated into the potting medium (30). Four weeks after seeding, DL-β-amino-*n*-butyric acid (BABA), which is a chemical elicitor of acquired resistance, was sprayed onto half of the nonbacterized plants as a positive control. One week later, all plants were challenge-inoculated with *Phytophthora infestans*. The resulting disease severity, measured as the percentage of leaves covered with late blight lesions, was significantly reduced by treatment with SE34. In a study on the microbial ecology of bacterial strains that elicit ISR (31), treatment of tomato with strain SE34 resulted in significant increases in plant height and overall plant weight compared with nonbacterized plants.

Publications by Enebak and Carey (7) and Enebak et al. (8) reported results with some *Bacillus* PGPR on loblolly pine (*Pinus taeda*) and slash pine (*Pinus elliottii* var. *elliottii*). All of the strains used in these studies had previously been found to elicit ISR on other plant species. In the first study (8), the effects of the bacteria on growth of the two pines were evaluated. Bacterial suspensions were applied at the time of seeding stratified pine seeds into standard planting containers used in the forestry industry. Treatment with *B. sphaericus* strain SE56 and *B. pumilus* strains INR7, SE34, SE49, SE52, and T4 significantly enhanced the germination rate of both pine species. Three months after treatment, the effect of bacterial inoculation on plant growth was evaluated. On loblolly pine, treatment with strain INR7 increased the mean number of branches per seedling. None of the strains significantly enhanced the length or weight of shoots or roots. On slash pine, the weight of shoots was increased by treatment with strains INR7 and SE49. In a subsequent study (8), all of the same strains, except strain T4, were evaluated for the capacity to elicit systemic protection on loblolly pine against *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*, which causes fusiform rust of southern pines. One month after planting, the systemic fungicide Bayleton (triadimefon) was applied to one group of plants as a positive control. Suspensions of *Cronartium quercuum* basidiospores from field-collected telia on water oak (*Quercus nigra*) were sprayed onto the pine seedlings at five different times. Six months after the final application, the incidence of fusiform rust was determined. The experiment was conducted in each of 2 years. In 1 year, the percentage of seedlings infected (disease incidence) was reduced from 25% for the nontreated control to 5% with Bayleton and 2.5 to 5% with strains INR7, SE34, and SE52. All strains, except SE49, resulted in significant reductions in disease incidence compared with the nontreated control in either one of the 2 years or in the pooled data from both years.

B. subtilis AF1 has been suggested to induce resistance against *Aspergillus niger* on peanut (*Arachis hypogaea*). Strain AF1 was originally isolated from soils suppressive to pigeon pea (*Cajanus cajan*) wilt, caused by *Fusarium udum* (19), and was found to cause lysis of *Aspergillus niger* and to reduce the incidence of crown rot of peanut caused by the pathogen (21). In a study on pigeon pea, Podile et al. (20) found that strain AF1 stimulated plant production of phenylalanine ammonia lyase and peroxidase, and based on this finding, they suggested that AF1 elicits ISR. In a follow-up study with peanut, Sailaja et al. (27) reported that inoculation of peanut seed with AF1 in soil containing *Aspergillus niger* resulted in a significant reduction in the incidence of crown rot of seedlings. This biological control was associated with induction of lipoxygenase activity in seedlings, suggesting that AF1 elicited ISR in peanut. Given that conclusive demonstration of ISR is considered to require the spatial separation of the pathogen from the eliciting bacterium, the results of Sailaja et al. (27) do not confirm that the biological control exhibited by AF1 is the result of ISR. This case demonstrates a difficulty in studying ISR elicited by root-colonizing bacteria against soilborne pathogens. When a bacterium has been confirmed to exhibit biological control against a soilborne pathogen, how does one investigate elicitation of ISR as a potential mechanism for the observed biological control? Should the current requirement for spatial separation of the eliciting bacterium and the pathogen be considered an absolute requirement? Should the demonstration that a bacterium elicits enhancement of defense-related compounds be considered sufficient to state that the strain elicits ISR? These questions should be discussed among researchers working on ISR elicited by rhizobacteria. Until there is a consensus on the answer to these questions, it appears that in order to conclude that a rhizobacterium elicits ISR, one must demonstrate that a systemic reduction in disease occurs and that there is spatial separation of the pathogen and the inducer.

Field reports of systemic disease protection. Advancing from greenhouse trials to field trials is an important step toward the goal of practical applications of ISR elicited by PGPR. Several of the strains of *Bacillus* spp. that demonstrated efficacy for reducing disease or promoting plant growth first in greenhouse trials have been examined under field conditions.

On sugar beet, *B. mycoides* strain Bac J, when sprayed onto leaves using a suspension of log 7.0 CFU/ml, significantly reduced the severity of Cercospora leaf spot on sugar beet in field trials conducted during six growing seasons (1). The reductions in disease severity compared with the nontreated control ranged from 38 to 91%. In 2 of the 6 years, the level of disease reduction afforded by treatment with Bac J was not significantly different to that afforded by triphenyltin hydroxide, which is the most commonly used fungicide for Cercospora leaf spot (1). As discussed previously, ISR was suggested as the mechanism of disease control in greenhouse tests that provided spatial separation of the pathogen and PGPR. However, such spatial separation was not provided in the field tests.

In a review of case studies of ISR elicited by rhizobacteria, Zehnder et al. (34) provided data from a field trial showing that *B. pumilus* strain INR7 reduced the incidence of cucurbit wilt disease on cucumber. The pathogen, *Erwinia tracheiphila*, is vectored by cucumber beetles, and the effect of ISR elicited by strain INR7 on beetles is discussed in the following section. In this study, bacteria were applied as seed treatments and drenches when preparing transplants. Treated and nonbacterized plants were transplanted into the field. In one of two test years, naturally occurring bacterial wilt was evident in the trials. The mean percentage of wilted vines (disease incidence) was significantly lower with an insecticide treatment and strain INR7 compared with that of the nonbacterized control in the middle of the growing season. The magnitude of protection afforded by treatment with strain INR7 was not significantly different from the protection from the insecticide control.

Reductions in disease incidence or severity caused by viruses have been confirmed in the field with the use of selected strains of ISR-eliciting *Bacillus* spp. In two field trials conducted in consecutive cropping seasons, Zehnder et al. (35) evaluated *B. subtilis* IN937b, *B. pumilus* SE34, and *B. amyloliquefaciens* IN937a for ISR against CMV on tomato. PGPR were applied as seed treatments and drenches at the time of transplanting to larger pots prior to transplanting in the field. CMV inoculum was applied to plants 1 week prior to transplanting in the field. Treatment with all three *Bacillus* strains resulted in significant reductions in the area under the disease progress curve (AUDPC) compared with that of the nonbacterized control, in both years of testing. Interestingly, ELISA values, which are an indication of viral titer within the plant, were significantly reduced by all three bacteria in the first year but were not affected by bacterial treatment in the second year. Hence, disease severity throughout the season was reduced even when virus titer in the plants was not changed by bacterial treatment. In both years, plant height at 30 days after transplanting was significantly enhanced by all bacterial treatments, and yield was significantly increased by all bacteria in the first but not in the second year.

In another viral study (17), various formulations of *B. amyloliquefaciens* IN937a, *B. subtilis* IN937b, and *B. pumilus* SE34 were tested in three field trials for the capacity to reduce incidence and severity of *Tomato mottle virus* (ToMoV) that is transmitted by whiteflies. In the field trials, PGPR were applied at the time of seeding to prepare transplants. The plots were inoculated with ToMoV by natural movement of viruliferous silver whitefly adults (*Bremisia argentifolii*) from adjacent plantings of ToMoV-resistant tomato germ plasm that was inoculated prior to transplanting into the field. Disease severity was monitored by a disease index; disease incidence was determined using Southern dot blot analysis; and yield was calculated by harvesting marketable and nonmarketable fruit twice from each field trial. In the first field trial (fall 1997), disease severity, compared with that of the nontreated control, was significantly reduced by two formulations of strains IN937a and IN937b, and disease incidence was significantly reduced by one formulation of IN937a and IN937b. The number of whitefly nymphs was significantly reduced, and marketable fruit yield was increased by one formulation of both PGPR strains. In the second trial (spring 1998), strain SE34 was used instead of strain IN937a. Significant reductions in the severity of ToMoV and in disease incidence resulted from one formulation of IN937b and two formulations of SE34. A significant increase in marketable fruit resulted at the first harvest with one formulation of IN937b. In the third trial in fall 1998, all three bacteria were tested. No bacterial treatment significantly reduced disease incidence or severity, and no treatment significantly increased yield, relative to the nontreated control.

Protection against angular leaf spot of cucumber via bacterial-induced ISR was first demonstrated by the group at Auburn University in greenhouse tests using gram-negative bacteria (12). When work at Auburn became concentrated on *Bacillus* spp., field trials were already underway, so greenhouse testing of the strains of *Bacillus* spp. was not conducted. In two field trials conducted in separate years, Raupach and Kloepper (23) reported that seed treatment of cucumber with *B. amyloliquefaciens* IN937a, *B. subtilis* GB03, and a mixture of the two strains resulted in significant increases in plant growth and reductions in disease severity. The length of the main runner (stem) was increased by all treatments, relative to the control, each year without fumigation of soil with methyl bromide. In the same plots, disease severity was significantly reduced by all three bacterial treatments. In a previous report, Wei et al. (29) evaluated *B. pumilus* INR7 in two field trials. In both trials, treatment with INR7 resulted in significant growth promotion evidenced by the length of the main runner and the number of leaves per plant, relative to the nontreated control. The severity of angular leaf spot following inoculation with *P. syringae* pv. *lachrymans*, and the severity of naturally occurring anthracnose, caused by *Colletotrichum orbiculare* (Berk. & Mont.) Arx, were significantly reduced by INR7 in each trial. The cumulative yield of marketable cucumber fruit was also significantly enhanced by INR7 in both field trials.

In similar field trials conducted on cucumber over several years, reduced severity and incidence of cucurbit wilt disease following treatment with several PGPR strains were noted by Wei (*unpublished data*). In these early studies, *Bacillus* spp. were isolated from a healthy cucumber plant within a heavily diseased field following surface disinfestation of the stem to isolate endophytic bacteria. Zehnder et al. (33) subsequently confirmed that strain INR7 elicited systemic protection in field trials against cucurbit wilt.

Before ending the summary of field trials, one case where ISR was not elicited, either by bacteria or by chemicals, should be noted. Based on the publications, summarized here, that several strains of *Bacillus* spp. can elicit ISR against many different pathogens on many hosts, Zhang et al. (40) reasoned that bacterial-elicited ISR could aid in management of late leaf spot of peanut, caused by *Cercosporidium personatum* (Berk. & M. A. Curtix) Deighton. Nineteen strains of bacilli, including the strains used in the previously discussed experiments where ISR was elicited by bacteria, were evaluated first in the greenhouse and then in the field as seed treatments. In the greenhouse trials, reductions in disease severity sometimes occurred with specific bacterial strains, but the response was not repeatable. Similarly, in the greenhouse, three chemical elicitors of ISR failed to protect plants. Some protection was seen in the greenhouse with (BABA) at a relatively high concentration, which is considered a result of elicitation of localized acquired resistance. Comprehensive field trials were conducted in each of 2 years. In these trials, all 19 bacteria were evaluated by application at two rates the first year. Three chemical elicitors of ISR and BABA, which elicits localized resistance, were also evaluated each year. In addition, some four-strain combinations of PGPR and BABA were evaluated in the field trials. Results revealed that some combinations resulted in significantly reduced disease severity at one, but not at the second, evaluation date. Collectively, the results suggest that peanut may not be systemically inducible by chemical or biological elicitors that trigger ISR in other crops. It should be noted, as discussed previously, that a different suggestion was made by Sailaja et al. (27), who suggested *B. subtilis* strain AF1 elicited ISR in peanut. However, as previously discussed, spatial separation of the bacterium and the pathogen was not maintained in this study, making it not possible to conclude that ISR was the mechanism of biological control.

Some other cases in which strains of *Bacillus* spp. elicited systemic protection in field trials are discussed in the following section on insect protection.

Reports of systemic insect protection. In addition to eliciting ISR against pathogens, protection against insects has been noted in studies where the disease is vectored by insects. In the field trials discussed above with ToMoV (17), the effects of PGPR treatments on the population of various developmental stages of the silver whitefly (*Bremisia argentifolii*) were determined. In the first trial in fall 1997, the number of whitefly nymphs was significantly reduced by one formulation of both *B. amyloliquefaciens* IN937a and *B. subtilis* IN937b. In the second trial, strains IN937b and *B. pumilus* SE34 resulted in significantly lower numbers of whitefly crawlers, nymphs, and pupae on tomato plants. In a third trial in fall 1998, no bacterial treatments significantly reduced numbers of any developmental stages of whitefly.

The most studied case of systemic protection by *Bacillus* spp. against insects is with ISR elicited against cucurbit wilt disease. The pathogen, *Erwinia tracheiphila*, is a vascular-wilt pathogen that infects the xylem of cucumber and other susceptible cucurbits. The pathogen is completely dependent for survival and transmission on the striped cucumber beetle (*Acalymma vittatum*

F.) and the spotted cucumber beetle (*Diabrotica undecimpunctata howardi* Barber). The finding, discussed above, that cucumber treated with INR7 exhibited reduced severity of cucurbit wilt in the field, led to the following question. Is ISR elicited by *Bacillus* spp. the result of protection against the insect vectors of the disease or only protection against the pathogen following transmission by cucumber beetles to the plant? Field and greenhouse studies were designed to answer the question. In a 2-year field study, treatment with strain INR7 resulted in reduced feeding of cucumber beetles (33). In both years of the study, the season-long average number of cucumber beetles per plant was significantly lower on plants treated with INR7 than on nonbacterized plants. Interestingly, on the sample date of both years when the maximum numbers of beetles were observed in the field plots (9 June 1993 and 25 May 1994), treatment with INR7 resulted in significantly fewer beetles per plant than treatment with the insecticide control (weekly sprays with esfenvalerate), which in turn had significantly fewer beetles than the nontreated control. Reduced beetle feeding on plants treated with INR7 was confirmed in subsequent greenhouse studies (32). Using a large cage in a cross shape, beetles were offered a choice of plants treated or not treated with INR7. Beetle preference for nontreated plants was evident within the first 24 h of releasing the beetles into the cage. After feeding for 17 days, beetle damage remained significantly lower on cotyledons and stems of plants treated with INR7 than on nontreated plants.

Elicitation of altered beetle behavior and feeding preferences in the field and greenhouse following seed treatment with *B. pumilus* INR7 was unexpected. As summarized by Zehnder et al. (32), cucumber beetle feeding behavior is influenced by a group of secondary plant metabolites called cucurbitacins. Cucurbitacins are bitter compounds that are toxic to most insects. The cucumber beetles consume high quantities of cucurbitacins without toxicity, apparently as an evolutionary adaptation that protects the cucumber beetles from predation. Cucumber beetles seek out cucurbitacins, and concentrations of 1 ng cause cucumber beetles to demonstrate arrested feeding behavior, whereby the beetles feed intensely on plants without moving from plant to plant. Hence, as an explanation for reduced beetle feeding on plants treated with INR7, Zehnder et al. (32) reasoned that elicitation of ISR by INR7 might be accompanied by reduced production of cucurbitacins by cucumber plants. Support for this hypothesis was found in a study (32) in which treatment of two cultivars of cucumber (one with constitutively high and one with low concentrations of cucurbitacin) with strain INR7 resulted in significantly reduced production of cucurbitacin. Collectively, the results from studies on cucumber beetles demonstrate that *Bacillus* spp. can elicit unexpected yet important physiological changes in plants.

Mechanisms of ISR by *Bacillus* spp. In addition to being of innate scientific interest, studies on mechanisms are suggested to be valuable in extension of PGPR-elicited ISR to practical agriculture or horticulture. It has been suggested that mixtures of PGPR strains with different mechanisms (22) might more reliably benefit plants than would individual PGPR strains. This suggestion is predicated upon finding differences in host responses to eliciting PGPR strains and subsequently demonstrating that mixtures of strains are compatible.

One aspect of mechanisms is to determine what compounds associated with plant defense against pathogens are produced during PGPR-elicited ISR. Elicitation of ISR in sugar beet by *B. mycoides* strain Bac J (1) and *B. pumilus* strains 203-6 and 203-7 (2) was associated with enhanced peroxidase activity and increased production of one chitinase isozyme and two isozymes of β-1,3-glucanase. In the tobacco blue mold system, Zhang et al. (37) reported that plants treated with *B. pumilus* strain SE34 had greatly increased levels of salicylic acid, compared with that of nontreated plants or plants treated with two gram-negative bacteria, 1 day after challenge-inoculation with the pathogen.

Two cytological studies were conducted by Benhamou et al. (3,4) with *B. pumilus* strain SE34. In the first study (3), colonization of pea roots by *Fusarium oxysporum* f. sp. *pisi* was restricted to the epidermis and outer cortex of roots treated with SE34, whereas in nonbacterized roots, the pathogen extensively colonized the cortex, endodermis, and the paratracheal parenchyma cells and radiated toward the vascular stele. This reduction in fungal colonization by treatment with strain SE34 was associated with strengthening of the epidermal and cortical cell walls. In addition, roots treated with SE34 exhibited newly formed barriers beyond the site of fungal infection. These barriers were cell wall appositions that contained large amounts of callose and were infiltrated with phenolic compounds. Phenolic compounds, detected in transmission electron micrographs using gold-complexed laccase, accumulated in host cell walls, in intercellular spaces, and on the surface and inside of the invading pathogen's hyphae. In another study (4), the effect of SE34 alone or in combination with chitin on structural and cytochemical changes of tomato infected with *Fusarium oxysporum* f. sp. *radicis-lycopersici* was investigated. Treatment by strain SE34 reduced the severity of typical symptoms, including wilting of seedlings and numbers of brown lesions on lateral roots. This disease protection by strain SE34 was associated with more limited fungal colonization of roots, compared with that of nonbacterized control plants, and with marked changes in the host physiology. Physiological changes elicited by strain SE34 included an increase in host cell wall density, the accumulation of polymorphic deposits at sites of potential pathogen penetration, and the frequent occlusion of epidermal cells and intercellular spaces with an osmophilic, amorphous material that appeared to trap the invading fungal hyphae. The extent and magnitude of the host physiological changes elicited by strain SE34 were enhanced by the addition of chitosan. For example, a qualitative evaluation of labeling that resulted from using gold-complexed β-1,3-glucanase showed that higher amounts of β-1,3-glucans accumulated in roots from plants treated with strain SE34 plus chitosan compared with that of nonbacterized, chitosan-treated plants and nontreated plants. Interestingly, the overall chitin component of the invading pathogen was structurally preserved in roots treated with SE34 with or without chitosan at the time when hyphal degradation was apparent. This suggests that synthesis of chitinase in bacterial-treated roots is not an early event in the cascade of physiological steps in signal transduction that leads to induced resistance.

In the literature on elicitation of ISR by pseudomonads, the most often investigated component of mechanisms accounting for ISR is the study of signaling pathways in the plant (28). A few similar studies have been reported with *Bacillus* spp. that elicit ISR. In the tomato late blight system reported by Yan et al. (31), elicitation of ISR by *B. pumilus* SE34 on tomato lines with various mutations in signaling pathways was tested. ISR was elicited on *nahG* lines, which breakdown endogenous salicylic acid, but not in the ethylene-insensitive *NR/NR* line or in the jasmonic acid-insensitive *df1/df1* line. These results are consistent with studies on several strains of *Pseudomonas* spp. that elicit ISR (28) where ISR is typically independent of salicylic acid and does not result in activation of the *PR1a* gene that encodes production of the pathogenesis-related (PR) protein PR1a. Similar results were reported by Zhang et al. (37). In the tobacco blue mold system, SE34, as well as two strains of gram-negative bacteria elicited ISR on both wild-type and NahG transgenic tobacco lines evidenced by significant reductions in the severity of blue mold on bacterized plants compared with that on nonbacterized plants. The conclusion that SE34 elicits ISR via salicylic acid-independent pathways conforms to the model with ISR elicited by *Pseudomonas* spp. (28).

Different results were found with *B. pumilus* strain T4 (18) that elicited ISR in tobacco against wildfire, caused by *Pseudomonas syringae* pv. *tabaci*. In this system, a transgenic line of tobacco

(*Nicotiana tabacum* cv. Xanthi-nc) with a GUS reporter gene fused to the PR1a promoter had significantly reduced severity of wildfire compared with nonbacterized controls. Elicitation of ISR by strain T4 was associated with a significant increase in GUS activity in microtiter-plate and whole-plant bioassays. Hence, with strain T4, elicitation of ISR results in activation of *PR1a*, which is activated during salicylic acid-dependent signaling pathway (28).

In another study of signaling pathways, Ryu et al. (26) found different results with strain T4 in *Arabidopsis* spp. In this report, *B. pumilus* T4 and SE34, *B. amyloliquefaciens* IN937a, and *B. subtilis* GB03 were evaluated for elicitation of ISR against two different pathovars of *Pseudomonas syringae* (pvs. *tomato* and *maculicola*). Strains T4 and SE34 elicited ISR against both pathogens. Strains IN937a and GB03 did not elicit protection against either pathovar, although they demonstrated elicitation of ISR in other host–pathogen systems (Table 1 in literature citation 26). When tested on NahG plants, both T4 and SE34 elicited ISR against *Pseudomonas syringae* pv. *maculicola*. However, against *Pseudomonas syringae* pv. *tomato*, ISR was elicited by T4 but not by SE34. Hence, while a salicylic acid-independent pathway was dominant in the tests, a salicylic acid-dependent pathway appeared to be activated during ISR elicited by strain SE34 against one pathovar. Additional tests of T4 and SE34 on various mutant lines of *Arabidopsis* spp. (26) revealed that in agreement with results on signaling during ISR elicited by *Pseudomonas* spp., ISR elicited by strain SE34 was dependent on NPR1, jasmonic acid, and ethylene, and ISR elicited by strain T4 was dependent on ethylene. However, in contrast to results on signaling during ISR elicited by *Pseudomonas* spp., ISR elicited by strain T4 was independent of NPR1 and jasmonic acid.

Collectively, the results reported to date on signaling pathways of ISR elicited by *Bacillus* spp. demonstrate the following points. Some different pathways appear to be operable when ISR is elicited by selected strains of *Bacillus* spp. than when ISR is elicited by *Pseudomonas* spp. The specific signal transduction pathway that is promoted during ISR by *Bacillus* spp. depends on the strain, the host plant, and at least in one case, on the pathogen used on a given host.

Another way to examine mechanisms by which bacteria elicit ISR is to search for bacterial determinants of elicitation. With *Pseudomonas* spp., several investigations of bacterial determinants of ISR have been reported and are summarized by van Loon and Glick (28). In contrast, until 2003, we could find no corresponding investigations on bacterial determinants of ISR by *Bacillus* spp. In 2003, volatile extracts produced by two *Bacillus* spp. were found to be primary determinants of both plant growth promotion and elicitation of ISR (24,25). Ryu et al. (25) observed significant growth promotion of *Arabidopsis* spp. by *B. subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a in I-plates. I-plates are petri dishes with a raised plastic divider that separates agar on each half of the dish, thus preventing movement of soluble compounds. When either of the two bacteria were placed on one side of I-plates, *Arabidopsis* plants growing on the other side of the plate exhibited enhanced growth, presumably as a result of volatiles produced by the bacteria. Characterization of the volatile organic compounds (VOCs) produced by the bacteria, coupled with bioassays of fractions of VOCs, revealed that 2,3-butanediol and acetoin elicited plant growth promotion. The capacity of VOCs from strains GB03 and IN937a to promote plant growth was examined in several mutant lines of *Arabidopsis* sp., including cytokinin-, brassinosteroid-, and gibberellic acid-insensitive mutants as well as auxin-transport-deficient and cytokinin receptor-deficient mutants. VOCs from strain IN937a promoted growth of all mutants, whereas VOCs from strain GB03 did not promote growth on two lines: the cytokinin- and ethylene-insensitive mutant (double mutation) and the cytokinin receptor-deficient mutant. Hence, the cytokinin-signaling pathway appears to play a role in growth promotion with VOCs from strain GB03 but not IN937a. In a separate study (26), exposure of *Arabidopsis* spp. to VOCs from strains GB03 and IN937a resulted in a significant reduction in the severity of disease caused by *Erwinia carotovora* subsp. *carotovora*. Elicitation of ISR occurred with exposure to bacterial VOCs for as little as 4 days. When specific fractions of VOCs were tested for their capacity to elicit ISR, 2,3-butanediol was found to elicit ISR in a dose-dependent manner. Various mutant lines of *Arabidopsis* spp. were exposed to whole VOCs (not individual fractions). Mutant lines included a jasmonic acid-insensitive line (*coi1*), an ethylene-insensitive line (*ein2*), a salicylic acid-degrading line (NahG), a salicylic acid constitutively producing line (*crp*1), and a line that lacks synthesis of salicylate and activation of the regulatory gene *npr1*. VOCs from strain IN937a elicited ISR on all of these lines, whereas VOCs from strain GB03 elicited ISR on all lines except the ethylene-insensitive line. The importance of the ethylene-dependent signaling pathway for elicitation of ISR by VOCs from strain GB03 was confirmed in tests with GUS fusions as reporters for activity of the following genes: *PR1a* for salicylic acid, *PDF1.2* for jasmonic acid and ethylene, and *Jin14* for jasmonic acid. Exposure of plants to VOCs from strain GB03 increased GUS activity of *PDF1.2* but not of the other genes. This finding confirms that the ethylene pathway is involved with elicitation of ISR by VOCs from strain GB03. Collectively, the results of these two studies (18,19) demonstrate that (i) whole VOCs from two different *Bacillus* spp., which are known elicitors of ISR, elicit plant growth promotion and ISR; (ii) a specific component of these VOCs, namely 2,3-butanediol, elicits plant growth promotion and ISR; (iii) the signaling pathways for both growth promotion and ISR are different between the two strains; and (iv) with strain GB03, the signaling pathway for growth promotion (cytokinin-dependent) appears to be different from the pathway for ISR (ethylene-dependent).

Progress toward practical use in agriculture and horticulture. Extension of beneficial preliminary research with *Bacillus* spp. to practical use in agriculture and horticulture requires adaptive research aimed at overcoming innate variability of microbial-based disease management approaches. One approach to increasing efficacy of microbial inoculants is to use microbial treatments that deliver plant growth promotion along with disease protection, thereby increasing the probability of an economically significant return to the grower. In regards to promoting plant growth, *Bacillus* PGPR seem to have an advantage over pseudomonad PGPR among elicitors of ISR based on the frequent findings summarized above that when ISR was elicited on a given host, plant growth was enhanced. An example in which an agricultural product has been developed using the capacity of a strain of *Bacillus* spp. to elicit both plant growth promotion and ISR is with Yield Shield. Yield Shield consists of a spore preparation of the *B. pumilus* strain listed in this review as INR7 and designated by Gustafson, LLC as GB34 (details available online from the Gustafson website). The product received registration from the U.S. Environmental Protection Agency (EPA) in 2003 for use on soybeans to protect against *Rhizoctonia solani* and *Fusarium* spp. Seed treatment of soybean with Yield Shield and strain INR7 results in significant seedling growth promotion and in ISR, which is apparent both by a significant decrease in incidence and severity of *Rhizoctonia solani* inoculated onto stems at a point where INR7 does not colonize and by a systemic increase in lignification of plant cell walls (C.-M. Ryu and C.-H. Hu, *unpublished data*). It should be emphasized that Yield Shield is a unique case for a rhizobacterium that elicits ISR in that economically significant efficacy sufficient to warrant the cost of product development and EPA registration was shown for a single bacterial strain.

Another way to overcome the problem of variability from using microbial inoculants alone for disease control would be to com-

bine the use of microbial treatments with suboptimal concentrations of fungicides. We could find no direct reports on this subject using *Bacillus* spp. Nevertheless, indirect support for this approach can be found in the studies at Montana State University on ISR against Cercospora leaf spot of sugar beet (1). Table 2 in reference 1 shows that the level of disease protection resulting from treatment with the most widely used fungicide, triphenyltin hydroxide, was significantly greater than the protection resulting from *B. mycoides* strain Bac J in four of the six field trials. Combination of Bac J with the fungicide propiconazole resulted in a level of disease reduction that was statistically equivalent to that from treatment with triphenyltin hydroxide in five of the six field trials.

Given that there is a finite time period in which plants are protected against disease by any elicitor of induced resistance, it would seem reasonable to expect that booster treatments with elicitors, i.e., additional treatments of the elicitor during the growing season, could increase either the level or the duration of protection. Data in a report by Zehnder et al. (36) support this assertion. In field trials using *B. pumilus* T4 to elicit ISR against cucurbit wilt disease, caused by *Erwinia tracheiphila*, plants were treated with a soil drench of T4 at the time of seeding in the field and at weekly intervals for 4 weeks after seeding. The experiment was conducted with and without fumigation with methyl bromide. Without methyl bromide fumigation, the AUDPC was significantly reduced, compared with that of the nonbacterized control, by application of T4 with two and three booster treatments but not by treatment at the time of seeding alone or with one booster application. Plant growth was significantly enhanced by all applications of T4. In plots fumigated with methyl bromide, the AUDPC was significantly less and plant growth was significantly greater than the control with all applications of T4. Hence, in the presence of greater competition from soil microorganisms (without methyl bromide fumigation), T4 elicited ISR more effectively with multiple applications.

It has been suggested that mixing strains of biological disease control agents can increase the repeatability of efficacy. Support for this concept appears in some publications (22). Jetiyanon and Kloepper (10) extended a previous greenhouse study using mixtures of *Bacillus* strains that elicit ISR (9) to the field. Field tests were conducted in Thailand to find mixtures of ISR-eliciting bacteria that could protect several different hosts against multiple diseases, which are typical under the multi- or inter-cropping agricultural conditions that are predominant under Thai agricultural conditions. In tests conducted during the rainy and dry seasons, results revealed that some two-strain mixtures more consistently protected against disease than did a single strain. In both seasons, the mixture of *B. amyloliquefaciens* IN937a and *B. subtilis* IN937b significantly protected against all the tested diseases (southern blight of tomato, caused by *Sclerotium rolfsii*; mosaic of cucumber, caused by CMV; and anthracnose of long cayenne pepper, caused by *Colletotrichum gloeosporioides*). The mixture of strains IN937a and IN937b also resulted in significant yield increases of all crops during the rainy season and generally in significant promotion of midseason plant growth.

A case study in product development that uses the concepts of two-strain mixtures of *Bacillus* spp. that elicit ISR together with incorporation of the bacteria into the potting mix is found with the product BioYield by Gustafson (11). The concept was to develop a biological formulation consisting of components known to exert different mechanisms for control of diseases. The selected components and their mechanisms were chitosan for nematode control via promotion of indigenous soil predators and antagonists to root-knot nematodes, *B. subtilis* strain GB03 for control of soilborne pathogens via production of the antibiotic iturin, and one of several tested strains of *Bacillus* spp. that elicit ISR. Extensive greenhouse and field evaluations were made to determine the most efficacious formulation for repeatedly eliciting growth promotion and ISR (11,13,14). The most unexpected finding was that the three-component combination (chitosan plus two bacterial strains) exhibited a more consistent and greater magnitude of growth promotion and systemic protection against pathogens than did any of the individual components (11). Based on the results, the two-strain combination of *B. amyloliquefaciens* strain IN937a and *B. subtilis* strain GB03 was selected for product development. Since development of the initial product, BioYield concentrate, a flowable formulation that contains the two bacterial strains without chitosan as a carrier has been developed.

Conclusion. Several main points arise from this review of *Bacillus* spp. that elicit ISR and promote plant growth. First, specific strains of spore-forming *Bacillus* spp. can elicit ISR that results in reduction in disease severity by a broad range of pathogens. ISR is also well documented in the literature using fluorescent pseudomonad PGPR (28). Second, the same strains of *Bacillus* spp. that elicit ISR typically promote plant growth. With fluorescent pseudomonads, ISR is not as closely associated with growth promotion. Perhaps this difference indicates some fundamental differences in plant response to *Bacillus* PGPR in comparison to pseudomonad PGPR, but no specific studies comparing multiple aspects of plant response to the two groups of PGPR have been reported.

Another point of comparison is that the literature to date on *Bacillus* spp. as elicitors of ISR concentrates more on aspects of microbial ecology and the "robustness" of ISR and growth promotion under field conditions than does the literature on pseudomonads as elicitors of ISR. Correspondingly, the pseudomonad literature has many more studies on mechanisms, especially the molecular and physiological bases of ISR. The relative lack of literature indicating bacterial determinants for elicitation of ISR by *Bacillus* spp. in contrast to many such studies for pseudomonad PGPR is surely a result of the lack of basic molecular tools, such as broad host-range vectors, for *Bacillus* spp.

As stated in the introduction, practical use of PGPR-based products in the United States has advanced in the 1990s with an emphasis on strains of *Bacillus* spp. A search of competitive grant awards for rhizobacterial research in the United States and Europe demonstrates that most research is proceeding on pseudomonad systems, although these are generally not available for use by farmers. Given that one purpose of agricultural research is to support farmers' decisions of inputs and cultural practices for producing a crop, increasing the relative number of studies on mechanisms of ISR with *Bacillus* spp. is a desirable goal.

It is interesting to note that, while most cases cited here of elicitation of ISR by *Bacillus* spp. resulted from application of the bacteria to seeds or to the potting medium, when *Bacillus* spp. were applied to sugar beet and elicited ISR against Cercospora leaf spot (1,2), the bacteria were applied as foliar sprays. Combinations of seed treatments with foliar sprays should be evaluated as a potential means to enhance the level and repeatability of protection against pathogens.

Most of the studies on the signal transduction pathways of plants treated with *Bacillus* spp. that elicit ISR have concentrated on plant secondary metabolites that are related to protection against plant diseases. In the case of systemic protection against cucumber beetles, altered production of one group of secondary metabolites, cucurbitacins, was unexpectedly found. This finding suggests that *Bacillus* spp. can elicit physiological changes in the plant that have not yet been detected by researchers and yet may be important for the plant.

We believe that *Bacillus* spp. will be used more in production agriculture and horticulture based on recent progress shown in implementing microbial inoculants. As indicated previously, the use of selected mixtures of *Bacillus* strains, applications of booster inoculants, and formulation technologies are key approaches that have been shown to increase the efficacy of plant growth promotion and elicitation of systemic disease protection in

the field. As more such studies are done for specific crops in various geographical areas, adoption of microbial inoculants will most likely increase.

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