

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 45
ACT: 519 TRASLADOINFORME	

DIRECCIÓN DE NUEVAS CREACIONES

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Bogotá, D.C.,

Abogado(a)/Señor(a)
JAIME EDUARDO DELGADO VILLEGAS

Asunto: Radicación: 14-274210- -1
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 5429
 Folios: 45

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/EP2011/071418				
Fecha de Presentación Internacional	30/11/2011				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado 14-274210-00000-0000	12/Diciembre/2014
Reivindicaciones:	Radicado 14-274210-00000-0000	12/Diciembre/2014
Prioridad:	EP 10193335.6	01/Diciembre/2010
	US 61/419,438	03/Diciembre/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Presentación de solicitudes Fraccionarias (Art 36 D 486)

La presente solicitud corresponde a una divisional de la solicitud parental 13-133020-00000-0000.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. Con relación a la presente solicitud se estudiarán las reivindicaciones 1 a 12 de acuerdo con el radicado 14-274210-00000-0000.

Título de la solicitud: "Combinaciones de principios activos que comprenden piridietilbenzamidas y otros principios activos".

El problema planteado en esta solicitud consiste en la necesidad de suministrar combinaciones que comprendan ingredientes activos útiles para combatir plagas en plantas.

Para resolver este problema técnico la solicitud en estudio proporciona una combinación de fluoripam y *Bacillus subtilis* cepa GB03.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A01N31/08, 43/28, 57/32; A01P5/00, 7/04.

5. De la solicitud de patente

Título

El título no permite identificar el alcance de la invención ya que define de forma general las combinaciones en términos de su familia química o su actividad biológica, por lo anterior, se sugiere restringir el título incluyendo las características técnicas inventivas. Además, es necesario que el título permita distinguir la presente solicitud de la solicitud original y sus divisionales.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 se encabeza como una combinación entre los compuestos fluopiram y *Bacillus subtilis* cepa GB03, sin embargo, de acuerdo con los ejemplos sustentados lo definido corresponde a composiciones, por lo que, se sugiere cambiar el encabezado combinación por composición. Además, las reivindicaciones deben ser claras en cuanto al tipo producto que reclaman.

La reivindicación 2 no es clara porque reclama una procedimiento de preparación sin definirlo en términos de una serie lógica y ordenada de etapas, además, no

establece en estás las condiciones, ingredientes y parámetros que permiten alcanzar el objeto propuesto. Se sugiere hacer las aclaraciones necesarias.

Las Reivindicaciones 2-3 incluyen las expresiones “composiciones insecticidas y/o acaricidas y/o nematocidas” y “combatir plagas animales” que refieren el uso de la composición reivindicada, el cual no es una característica técnica que defina a la invención. Se sugiere eliminar dichas expresiones.

La reivindicación 3 no es clara porque define los excipientes en términos de su función dentro de la composición, se recuerda, que los excipientes se deben definir en términos de su nombre común y proporciones dentro de la composición, se sugiere hacer las aclaraciones necesarias.

La reivindicación 4 no es clara porque la expresión “por ejemplo” precediendo una característica no es limitativa, porque hace opcional a la característica y no precisa el alcance de la reivindicación. Se sugiere incluir una expresión concreta de acuerdo con los ejemplos sustentados.

La reivindicación 10 no es clara porque reclama la inclusión de arcillas modificadas sin caracterizarlas, se sugiere hacer las precesiones necesarias.

Las reivindicaciones 2-12 no son concisas porque las definiciones de los excipientes son generales, por lo que se sugiere restringir la solicitud a una razonable generalización de los excipientes probados, es decir, que se encuentran debidamente sustentados a través de ejemplos concretos de acuerdo con lo presentado en la tabla No 1.

Tabla No. 1 Excipientes de las composiciones soportadas.

Excipientes	Ejemplos probados
Tensioactivos	No sustenta
Diluyentes	No sustenta
Disolventes	Acetona, dimetilformamida
Colorantes	No sustenta
Humectantes	No sustenta
Dispersantes y/o emulsionantes	No define (alquilarilpoliglicoléter)
Antiespumante	No sustenta
Conservante	No sustenta
Espesante	No sustenta
Adhesivos	No sustenta
Gibelinas	No sustenta

Las reivindicaciones 2-12 reclaman que la composición incluye tensioactivos, diluyentes, colorantes, humectantes, antiespumantes, conservantes, espesantes, adhesivos y gibelinas, sin embargo, dentro del capítulo descriptivo no se definen ejemplos concretos de composiciones que comprendan los mencionados excipientes, se aclara, que la mera mención de la posibilidad de incluir unos excipientes seleccionados de una extensa lista no es suficiente soporte, las composiciones se deben definir en términos de sus ingredientes (DCI/IUPAc para los activos y nombre común para los excipientes) y proporciones, por lo anterior, se sugiere eliminar de las mencionadas cláusulas los excipientes no sustentados.

Debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito para la cepa GB03 de *Bacillus subtilis*.

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de la cual se invoca prioridad, es decir, diciembre 01 de 2010. Consultadas las fuentes de información con que se cuenta, se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US20100249193	“Nematicidal, Insecticidal and Acaricidal Active Ingredient Combinations Comprising Pyridylethylbenzamides and Insecticides”	30/Septiembre/2010
D2	US5215747	“Composition and method for protecting plants from phytopathogenic fungi”	01/Junio/1993

El documento D1⁽¹⁾ divulga combinaciones que comprenden fluopiram útiles en el control de plagas producidas por insectos, ácaros y nematodos en suelo, hojas y semillas (Resumen y Pág. 11, Rv. 1).

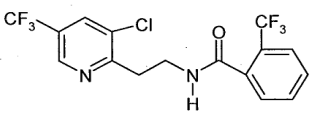
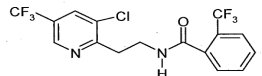
El documento D2⁽²⁾ divulga composiciones que comprenden *Bacillus subtilis* y al menos un compuesto fungicida, útiles para proveer protección a las plantas contra los hongos (Resumen y Col. 8, Rv. 1).

7. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en “La reivindicación 1 consiste en la “Combinaciones de principios activos que comprenden fluopiram y *Bacillus subtilis* cepa GB03”.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Combinación que comprenden:	Combinación de ingredientes que comprende: (Pág. 11, Rv. 1)	Composiciones que comprenden: (Col. 8, Rv. 1)
 (Fluopiram)	 (Fluopiram) (Pág. 11, Rv. 1)	Fungicida (Col. 8, Rv. 1)

1 . [www.espacenet/ US20100249193](http://www.espacenet/US20100249193)

2 . [www.espacenet/ US5215747](http://www.espacenet/US5215747)

<i>Bacillus subtilis</i> cepa GB03	<i>Bacillus firmus</i> (Pág. 11, Rv. 1)	<i>Bacillus subtilis</i> (Col. 8, Rv. 1)
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Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque en el caso del documento D1 divulga combinaciones que comprenden fluopiram útiles en el control de plagas producidas por insectos, ácaros y nematodos en suelo, hojas y semillas (Resumen y Pág. 11, Rv. 1). Por su parte, el documento D2 divulga composiciones que comprenden *Bacillus subtilis* y al menos un compuesto fungicida, útiles para proveer protección a las plantas contra los hongos (Resumen y Col. 8, Rv. 1).

La diferencia entre la combinación, definida en la reivindicación 1 y la combinación revelada en el documento D1 consiste en que la combinación reclamada incluye al microorganismos *Bacillus subtilis* cepa GB03, mientras que la combinación divulgada en el documento D1 incluye al microorganismo *Bacillus firmus*.

El efecto que logra el utilizar al *Bacillus subtilis* cepa GB03 en la combinación consiste en que se aumenta la capacidad de combate de ésta contra las plagas.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo proporcionar combinaciones que comprendan Fluopiram útiles para el control de las infestaciones de plagas en plantas”.

Sin embargo, la combinación de un agente químico fungicida con *Bacillus subtilis* cepa GB03 útil para tratar infestaciones de plagas en plantas, está divulgada en el documento D2 que enseña composiciones que comprenden *Bacillus subtilis* y al menos un compuesto fungicida, útiles para proveer protección a las plantas contra los hongos (Resumen y Col. 8, Rv. 1).

Además, es parte del conocimiento de la persona normalmente versada en la materia que el microorganismo *Bacillus subtilis* cepa GB03 promueve el crecimiento de las plantas ya que aumenta la resistencia de éstas al ataque de los fitopatógenos a través de la producción de elicitores como 2,3 butanediol, de acuerdo con lo divulgado en los documentos “Interactions of *Bacillus* spp. and plants—With special reference to induced systemic resistance (ISR)”⁽³⁾, “Dynamic communication between plants and rhizobacteria via volatile signals”⁽⁴⁾ y “Mixtures of Plant Growth-Promoting Rhizobacteria Enhance Biological Control of Multiple Cucumber Pathogens”⁽⁵⁾.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría dentro de la combinación divulgada en el documento D1 al microorganismos *Bacillus subtilis* cepa GB03 de acuerdo con lo revelado en el documento D2, para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

3 . <http://www.sciencedirect.com/science/article/pii/S0944501308000566>

4 . <http://www.bashanfoundation.org/kloepper/kloepervolatile.pdf>

5 . <http://www.ag.auburn.edu/users/kloepjw/pdfpapers/raupachkloepper8811115811641998.pdf>

Puesto que las reivindicaciones dependientes 2-12 no mencionan características técnicas esenciales acerca de la combinación, tampoco se pueden considerar inventivas.

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US20100249193	1-12	Nivel inventivo
D2	US5215747	1-12	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2015-05-26

Elaboró: German Antonio Clavijo Cohen
Revisó: Jeny Paola Villate Becerra

INFORME DE BÚSQUEDA DE PATENTES PARA EL No de Radicación: 14-274210						
CRITERIOS PARA LA BÚSQUEDA (anexar pantallazo o reporte en PDF, junto con los boleanos utilizados)						
Palabras clave utilizadas		- Microbial disruptors - <i>Bacillus subtilis</i> strain GB03 -Bacillus subtilis strain GB03 and fungicide - Bacillus subtilis strain GB03 and insecticide				
CLASIFICACIONES INTERNACIONALES UTILIZADAS PARA LA BÚSQUEDA		A01N31/08, 43/28, 57/32; A01P5/00, 7/04				
BASES DE DATOS CONSULTADAS.						
Google Patents						
WIPO						
Spacenet						
INFORMES DE EXÁMENES REALIZADOS EN OTRAS OFICINAS (documentos patentes, literatura no patente etc...)						
Oficina	Fecha del examen o de reporte de búsqueda de documentos	Anterioridades encontradas para el examen o reportados en el examen de búsqueda(relacionarlos todos)	Reiv. afectadas	RELEVANCIA (requisito que afecta)	FUERON TENIDOS EN CUENTA PARA EL EXAMEN EN LA OFICINA COLOMBIANA	
					SI	NO
EUROPEA	01/06/2013	WO2010108616	1-10	Novedad y Nivel Inventivo	X	
DOCUMENTOS ADICIONALES ENCONTRADOS POR EL EXAMINADOR DE ACUERDO A LOS CRITERIOS DE BÚSQUEDA.						
Interactions of Bacillus spp. and plants–With special reference to induced systemic resistance (ISR)						
Dynamic communication between plants and rhizobacteria via volatile signals						
Mixtures of Plant Growth-Promoting Rhizobacteria Enhance Biological Control of Multiple Cucumber Pathogens						
BÚSQUEDA DE SECUENCIAS						
Número de depósito / patente y N° de secuencia % de identidad						
SEQ ID NO (de la solicitud)	NCBI	The Lens	EMBL-EBI	DDJB	Genome Quest	
	No aplica					

CRITERIOS DE BÚSQUEDA / UNIDAD DE INVENCION (Pantallazos)

No aplica



Interactions of *Bacillus* spp. and plants – With special reference to induced systemic resistance (ISR)

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KEYWORDS

Aerobic endospore-forming bacteria (AEFB);
Induced systemic resistance (ISR);
Bacillus spp.;
Biocontrol;
Volatile organic compounds (VOCs)

Summary

Biological control of soil-borne pathogens comprises the decrease of inoculum or of the disease producing activity of a pathogen through one or more mechanisms. Interest in biological control of soil-borne plant pathogens has increased considerably in the last few decades, because it may provide control of diseases that cannot or only partly be managed by other control strategies. Recent advances in microbial and molecular techniques have significantly contributed to new insights in underlying mechanisms by which introduced bacteria function. Colonization of plant roots is an essential step for both soil-borne pathogenic and beneficial rhizobacteria. Colonization patterns showed that rhizobacteria act as biocontrol agents or as growth-promoting bacteria form microcolonies or biofilms at preferred sites of root exudation. Such microcolonies are sites for bacteria to communicate with each other (quorum sensing) and to act in a coordinated manner. Elicitation of induced systemic resistance (ISR) by plant-associated bacteria was initially demonstrated using *Pseudomonas* spp. and other Gram-negative bacteria. Several strains of the species *Bacillus 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

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damping-off, blue mold, and late blight diseases. This progress will lead to a more efficient use of these strains which is worthwhile approach to explore in context of biocontrol strategies.

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Introduction

Plant roots release substantial amounts of C- and N-containing compounds into the surrounding soil. Microorganisms are attracted to this nutritious environment and use the root exudates and lysates for growth and multiplication on the surface of root and in the adjacent rhizosphere soil. The rhizosphere is a densely populated area in which the roots must compete with the invading root systems of neighboring plant species for space, water, and mineral nutrients, and with soil-borne microorganisms, including bacteria, fungi, and insects feeding on an abundant source of organic material (Ryan and Delhaize, 2001). Thus, root–root, root–microbe, and root–insect communications are likely continuous occurrences in this biologically active soil zone, but due to the underground nature of roots, these intriguing interactions have largely been over-looked. Root–root and root–microbe communication can either be positive (symbiotic) to the plant, such as the association of epiphytes, mycorrhizal fungi, and nitrogen-fixing bacteria with roots; or negative to the plant, including interactions with parasitic plants, pathogenic bacteria, fungi, and insects. Thus, if plant roots are in constant communication with symbiotic and pathogenic organisms, how do roots effectively carry out this communication process within the rhizosphere? Plant growth-promoting rhizobacteria (PGPR) are

defined as root-colonizing bacteria (rhizobacteria) that exert beneficial effects on plant growth and development. Root colonization comprises the ability of bacteria to establish on or in the plant root, to propagate, survive and disperse along the growing root in presence of the indigenous microflora. Rhizobacteria are considered as efficient microbial competitors in the root zone. Representatives of many different bacterial genera have been introduced into soils, onto seeds, roots, tubers or other planting materials to improve crop growth. These bacterial genera include *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Frankia*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Thiobacillus*, and many others. In addition to the promotion of plant growth, PGPR are also employed for controlling plant pathogens, enhancing efficiency of fertilizers, and degrading xenobiotic compounds (rhizoremediation) (Kloepper et al., 2004a).

Because of the rapid consumption of the nutrients, bacterial growth in the rhizosphere remains nutrient-limited where roots are seldom colonized for more than about 15% of their surface area. The rhizosphere microflora plays an important role in plant development and acclimation to environmental stresses. Since the rhizosphere microflora is extremely diverse, a dynamic interplay between the members of the microbial community occurs which is mediated by synergistic and antagonistic

interactions within the limits of the nutrients available. Both Gram-negative and -positive bacteria, including plant-pathogenic bacteria such as *Erwinia* spp., *Pseudomonas* spp., and *Agrobacterium* spp., possess quorum sensing (QS) system that control the expression of several genes required for pathogenicity (Fray, 2002).

QS is a form of cell–cell communication between bacteria mediated by small diffusible signaling molecules (auto-inducers); these include acylated homo-serine lactones (AHLs) for Gram-negative bacteria and peptide-signaling molecules for Gram positive bacteria. Upon reaching a threshold concentration at high population densities, an auto-inducer then activates transcriptional activator proteins that induce specific genes. In addition, signals are exchanged between fungi and bacteria and plant roots which reflect a highly dynamic belowground communication network (Figure 1).

PGPR can suppress diseases through antagonism between bacteria and soil-borne pathogens, as well as by inducing a systemic resistance in the plant against both root and foliar pathogens. The induced resistance constitutes an increase in the level of basal resistance to several pathogens simultaneously, which is of benefit under natural conditions where multiple pathogens exist (van Loon and Glick, 2004).

Plants possess a range of active defense apparatuses that can be actively expressed in response to biotic stresses (pathogens and parasites) of various scales (ranging from microscopic viruses to phytophagous insect). The timing of this defense response is critical and reflects on the difference

between coping and succumbing to such biotic challenge of necrotizing pathogens/parasites (Choudhary et al., 2007). Pathogenic microorganisms affecting plant health are major and chronic threats to food production and ecosystem stability worldwide. Crop rotation, breeding for resistant plant varieties and the application of chemical plant protection means are insufficient to control root diseases of important crop plants (Johri et al., 2003).

Despite inconsistency in field performance, biological control is considered as an alternative or a supplemental way of reducing root diseases in agroecosystem (Sharma and Johri, 2003). The widely recognized mechanism of biocontrol mediated by PGPR is competition for an ecological niche/substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens. Recently, research on mechanisms of biological control by PGPR revealed that several PGPR strains protect plants against pathogen infection through induction of systemic resistance, without provoking any symptoms themselves. More recently, PGPR-mediated induction of ISR has been reported for several other plant-pathogen systems (Table 1).

In recent years, several PGPR-based products became commercially available in the United States that used as biological control agents (BCAs) (Table 2). 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

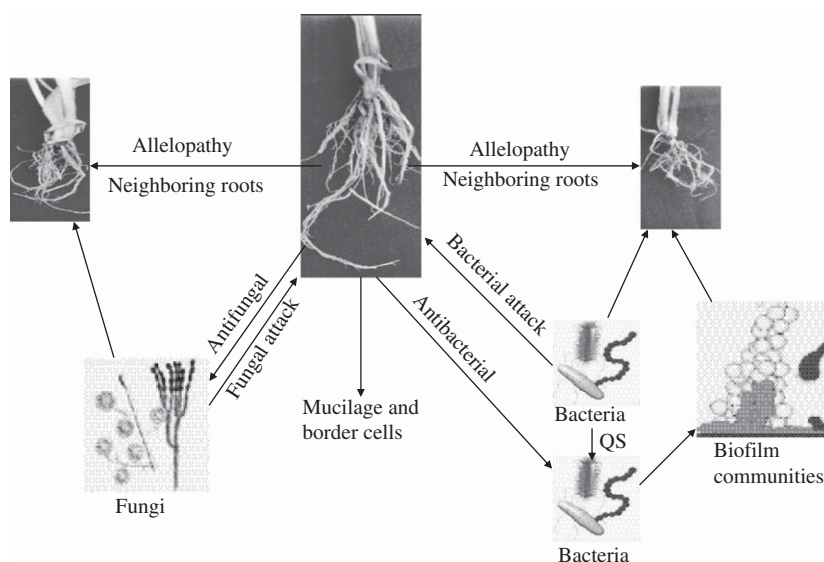


Figure 1. Schematic representation of complex interactions mediated by root exudates that take place in the rhizosphere between plant roots and other organisms. QS, quorum sensing. Source: Walker et al. (2003).

Table 1. PGPR-mediated induction of ISR in different plant species

Bacterial strain	Plant species	Pathogen	Elicitors	References
<i>B. amyloliquefaciens</i> IN 937a	<i>Arabidopsis</i>	<i>Erwinia carotovora</i>	2,3-Butanediol	Ryu et al. (2004)
<i>B. subtilis</i> GB03	<i>Arabidopsis</i>	<i>Erwinia carotovora</i>	2,3-Butanediol	Ryu et al. (2003)
<i>B. pumilus</i> T4	<i>Arabidopsis</i>	<i>Pseudomonas syringae</i> pv. <i>Maculicola</i>	SA	
<i>B. pumilus</i> SE34	<i>Arabidopsis</i>	<i>P. syringae</i> pv. <i>maculicola</i>	SA	Ryu et al. (2003)
	Tobacco	<i>Peronospora tabacina</i>	SA	Zhang et al. (2002)
<i>P. aeruginosa</i> 7 NSK2	Bean	<i>P. syringae</i>	SA	De Meyer et al. (1999a)
	Tobacco	TMV	SA	De Meyer et al. (1999b)
	Tomato	<i>Botrytis cinerea</i>	Pyochelin and Pyocyanin	Audenaert et al. (2002)
	Tomato	<i>Meloidogyne javanica</i>		Siddiqui and Saukat (2004)
<i>P. fluorescens</i> CHAO	<i>Arabidopsis</i>	<i>Peronospora parasitica</i>	2,4 DAPG	Iavicoli et al. (2003)
	Tobacco	TNV	Siderophore	Maurhofer et al. (1994)
	Tomato	<i>Meloidogyne javanica</i>	2,4 DAPG	Siddiqui and Saukat (2004)
<i>P. fluorescens</i> Q2-87	<i>Arabidopsis</i>	<i>P. syringae</i> pv. <i>Tomato</i>	2,4 DAPG	Weller et al. (2004)
<i>P. fluorescens</i> WCS 374	Radish	<i>Fusarium</i> sp.	Siderophore and Fe-regulated compounds	Leeman et al. (1996)
<i>P. fluorescens</i> WCS 417	Carnation	<i>F. oxysporum</i>	LPS	van Peer and Schippers (1992)
	Radish	<i>Fusarium</i> sp.	Fe-regulated compounds	Leeman et al. (1996)
<i>P. putida</i> WCS 358	<i>Arabidopsis</i>	<i>P. syringae</i>	LPS, Siderophore, Flagella	Meziane et al. (2005)
	Bean	<i>P. syringae</i>	LPS, Siderophore	Meziane et al. (2005)
<i>P. fluorescens</i> GRP ₃	Tomato		LPS, Siderophore	Meziane et al. (2005)
	Rice	<i>Rhizoctonia</i> sp.	Siderophore	Pathak et al. (2004)

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. Compared to plant growth-promoting *Pseudomonas* rhizobacteria, relatively little is known about the lifestyle of plant-associated *Bacillus* spp., which was originally considered as typical soil bacteria, despite their well-established advantages for beneficial action on plant growth and biocontrol (Kloepper et al., 2004a).

The plant root-colonizing *Bacillus amyloliquefaciens* strain FZB42 is a naturally occurring isolate and has the ability to stimulate plant growth and suppress plant pathogens (Idris et al., 2002). The effect of long-living spore formulations of the *Bacillus* strain FZB24 and FZB42 produced and commercialized by Abitep GmbH Berlin was tested in extended and long lasting field trials on potatoes under practical farming conditions. Efficacy in

plant-growth promotion was determined in manifold field trials conducted in different farming areas in North East (Federal State Brandenburg) and North West Germany (Lüben) and also in Koolen (Netherlands) using either water dispersible granule or liquid formulations of the bacilli spores. Forty-eight field trials performed with FZB24 under standard conditions (250 g/ha) during 1995–1998 resulted in an increase of tuber yield of 8.3% as the mean value. A large number of field trials on potatoes conducted in 2002, 2003, and 2004 confirmed that FZB strains in fact enhance productivity of about 7.5–10% (mean value of 87 independent trials). Best results were obtained if application of bacilli was combined with use of fungicides as Risolex or Monceren. In such cases an increase of tuber yield up to 40% was obtained. Moreover, a significant reduction of chemical fertilizer was also achieved. In addition, biocontrol activity directed against stem canker and black

Table 2. Examples of PGPR-based commercial products in USA

Product (brand name)	Biocontrol agent	Target pathogen/disease
Green Releaf	<i>Bacillus licheniformis</i> SB3086	Fungal species especially those causing leafspot and blight diseases
EcoGuard	<i>B. licheniformis</i> SB3086	Dollar spot
GB34 Biological Fungicide	<i>B. pumilus</i> GB34	<i>Rhizoctonia</i> and <i>Fusarium</i>
Ballad	<i>B. pumilus</i> QST2808	Soybean rust
Companion	<i>B. subtilis</i> GB03	<i>Rhizoctonia</i> , <i>Pythium</i> , <i>Fusarium</i> , and <i>Phytophthora</i>
Frostban A	<i>Pseudomonas fluorescens</i> A506 and 1629RS, <i>P. syringae</i> 742RS	Frost forming bacteria
Frostban B	<i>P. fluorescens</i> A506	Frost forming bacteria
Frostban C	<i>P. syringae</i> 742RS	Frost forming bacteria
Frostban D	<i>P. fluorescens</i> 1629RS	Frost forming bacteria
BlightBanA506	<i>P. fluorescens</i> A506	Frost forming bacteria, <i>Erwinia amylovora</i>
AtEze	<i>P. chlororaphis</i> 63-28	Wilt diseases, stem and root rots
Bio-save 10LP, 110	<i>P. syringae</i>	<i>Botrytis cinerea</i> , <i>Penicillium</i> spp., <i>Mucor pyroformis</i> , <i>Geotrichum candidu</i>

Source: APS Biological Control Committee-<http://www.oardc.ohio-state.edu/apsbcc>.

scurf of potatoes caused by *Rhizoctonia solani* and common potato scab caused by *Streptomyces scabies* was registered. Whole genome sequencing of environmental *B. amyloliquefaciens* strain FZB42 is now completed. Preliminary analysis ruled out that about 8.5 percentage of its 3.9 Mbp genome is devoted to synthesis of antibacterial and antifungal acting polyketides and lipopeptides, suggesting that its high biocontrol activity is at least partly due to its impressive genetic capacity to synthesize nonribosomally such bioactive metabolites (Chen et al., 2007).

In this review, we summarize research on *Bacillus* PGPR which elicits ISR and plant growth.

Aerobic endospore-forming bacteria (AEFB) in rhizosphere

Diverse populations of AEFB, viz., *Bacillus* spp., are denizen of agricultural fields and may directly and indirectly contribute to crop productivity. Common metabolic traits important to their survival include production of a multilayered cell wall structure, formation of stress resistant-endospores, and secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes. In addition, significant variation exists in other key traits, including nutrient utilization, motility, and physiochemical growth optima. Quantitative and qualitative variations in these traits allow for these bacteria to inhabit diverse niches in agro-ecosystems (McSpadden Gardener, 2004).

Abundance and distribution of *Bacillus* spp. in agro-ecosystem

Multiple *Bacillus* spp. can be readily cultured from both bulk and rhizosphere soil on solid medium where cultivable counts of these bacteria generally range from log 3 to log 6 cells per gram fresh weight, with soil counts typically exceeding those obtained from the rhizosphere (Vargas-Ayala et al., 2000). Multiple isolates of phenetically and phylogenetically similar species that related to *Bacillus subtilis* and *Bacillus cereus* have been recovered employing standard isolations on complex media. Most distinctive among these morphologically is *Bacillus mycoides*, which often confound attempts to accurately enumerate cultured populations by virtue of their rapid mycelial-like growth patterns on solid media (McSpadden Gardener, 2004).

Cultivation-independent analyses of soil DNA have confirmed the presence of uncultured diversity in *Bacillus* rRNA lineages. Several reports indicated that the dominant *Bacillus* sequences present in a different soil are not the same as those present in easily cultured isolates. Interestingly, the substantial effort leading to the isolation of this previously uncultured lineage (referred to as DA001) also led to the isolation of even more microdiversity (phylogenetically similar but physiologically dissimilar) that had not been previously directly detected in DNA clone banks of sequences obtained from the same soil (Felske et al., 2003).

The phylogenetic relatedness of three *Bacillus* species, viz., *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains was initially established by both phenotypic and genotypic characterization (Priest, 1993). More recently, the degree of homogeneity of this group has been further established by analyses of multiple gene loci and amplified fragment length polymorphism (AFLP) (Ticknor et al., 2001). Different strains of *B. subtilis* could be differentiated employing phenotypic and genotypic approaches, but this is not a case for biocontrol functions. Variation in *cry* genes (which express the crystal proteins that are toxic to invertebrate species) that recovered from *B. thuringiensis* is well known and needed to explore continuously (Stahly et al., 1992).

Phenotypic characterizations of culture isolates have revealed a tremendous degree of ecologically relevant diversity at the species level in the AEFB, which is case study for *Bacillus sphaericus* and *B. thuringiensis*, both of which are more capable of saprophytic growth (Stahly et al., 1992; Priest, 1993). Several species were initially defined based on the extreme physico-chemical conditions under which they were first isolated, viz., *B. psychrophilus* together with obligate extremophiles (viz., *B. stearomophilus*) (Priest, 1993). Furthermore, multiple species can be recovered as epiphytes and endophytes of plants and animals along with foodstuffs and composts derived from them (Stahly et al., 1992). The rich variety of organic substrates and micro-niches present in such environments support a complex milieu of microbial species wherein multiple species of *Bacillus* inhabit them.

Recently, signature sequences (e.g., 16S rDNA) have been employed to characterize the relative distribution of *Bacillus* spp. between soils and plant tissues. Previously, DNA-based studies of bacteria, including AEFB, revealed relatively little variation in the abundance of soil microbes, even though distinct differences in community structure could be observed in adjacent sites differing in land management practices. At the species level, most *Bacillus* are globally distributed and this ubiquitous occurrence of more defined subspecies of *B. subtilis* and *B. cereus* with the capacity to suppress phytopathogens has also been reported (Priest, 1993; Pinchuk et al., 2002). Researchers reported highly significant differences in the total and relative abundance of *Bacillus*-like sequences amplified from bulk soil and crop roots and this is because of large differences in the relative abundance of terminal restriction fragments (TRFs). This is consistent with analyses of whole bacterial communities that showed significant

distinctions between soil and rhizosphere communities (Smalla et al., 2001).

In addition, Chen et al. (2007) revealed the complete genome sequence of *B. amyloliquefaciens* FZB42, and through comparison with the domesticated strain *B. subtilis* 168, highlighted genes that may contribute to its plant-associated life style. Its 3918-kb genome, containing an estimated 3693 protein coding sequences, lacks extended phage insertions, which occur ubiquitously in the closely related *B. subtilis* 168 genome. The genome of *B. amyloliquefaciens* FZB42 genome reveals an unexpected potential to produce secondary metabolites, including the polyketides bacillaene and difficidin. More than 8.5% of the genome is devoted to synthesizing antibiotics and siderophores by pathways not involving ribosomes. The ability of *B. amyloliquefaciens* FZB42 to efficiently colonize phytosphere is a prerequisite for phytostimulation, which linked to the capability to form sessile, multicellular communities, viz., biofilms. The genome of *B. amyloliquefaciens* FZB42 contains the complete set of genes implicated in biofilm- and fruiting-body formation in *B. subtilis*, including the 15-gene exopolysaccharide (EPS) operon *epsA-O*, apparently required for producing an EPS that holds chains of cells together in bundles.

Analysis of 16S rRNA sequence of *B. amyloliquefaciens* FZB42, indicates that it is closely related, but not identical, to the *B. amyloliquefaciens* type strain DSMZ7 (Idris et al., 2002). A phylogenetic tree constructed from the tetranucleotide usage patterns that derived from genomes of previously sequenced *Bacillus* strains confirmed that *B. amyloliquefaciens* FZB42 represents a separate branch, clearly distinct from *B. subtilis* 168 (Chen et al., 2007).

Culture-based studies on bacterial endophytes indicated that *Bacillus* spp. were present inside various plant tissues but in low abundance. *B. megaterium* has been reported to be one of the most abundant populations of culturable AEFB present in the soybean rhizosphere whereas abundance of *B. pumilus* and *B. subtilis* reported from the phyllosphere of soybeans (Arias et al., 1999).

Bacillus and plant health

A number of *Bacillus* strains express activities that suppress necrotizing pathogens/parasites or otherwise promote plant growth. Improvements in plant health and productivity are mediated by three different ecological mechanisms:

(i) antagonism of pest and pathogens, (ii) promotion of host nutrition and growth, and (iii) stimulation of plant host defenses.

Antagonism of pest and pathogen populations by AEFB takes several forms wherein species are pathogens of insects or nematodes (Stahly et al., 1992). The most studied of the insect pathogens are those classified as *B. thuringiensis* which is distinguished from the common saprophytic species *B. cereus* by the occurrence of plasmids that encode pathogenicity factors that make the strains very prone to pathogenic against various invertebrates together with nematodes. *B. sphaericus* is pathogenic to various Diptera species. However, it is more effective at controlling insects that bite animals and humans rather than those that damage crops. Several species of *Bacillus* are known to produce toxins that are inhibitory to the growth and/or activities of fungal and nematode pathogens of plants wherein most thoroughly studied species include *B. subtilis* (Pinchuk et al., 2002). In addition, a number of studies have reported direct antagonism by several other species that include *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, and *B. pumilus* together with isolates of unidentified species from the genus (Yu et al., 2002).

Catabolic enzymes (viz., proteases, chitinases, and glucanases), peptide antibiotics, and small molecules can be secreted by various species and many all contribute to pathogen suppression. Peptide antibiotics and several other compounds toxic to plant pathogens have been recovered from several *Bacillus* strains (Yu et al., 2002). *B. subtilis* strains that produce the lipopeptide antibiotics iturin A and surfactin could suppress damping-off in tomato whereas zwittermicin A from *B. cereus* have been correlated to suppression of damping-off in alfalfa. Antagonism also plays an important role, which involves competition for niche space and nutrients with other chemoheterotrophs in the phytosphere wherein motile and chemotactic strains of *B. megaterium* were shown to better colonize roots and suppress *Rhizoctonia solani* (Zheng and Sinclair, 2000).

Bacillus populations may also promote plant health by stimulating the plant host or mutualistic symbionts. Induction of host resistance pathways whether locally and systemically, has been reported for several isolates of *B. amyloliquefaciens*, *B. cereus*, *B. sphaericus*, *B. subtilis*, *B. mycoides*, and *B. pumilus*. Plant hosts may also be affected by hormones known to be produced by various microbial species including *B. subtilis* (Priest, 1993). Such compounds (e.g., auxins, gibberellins, and cytokinins) mediate processes that include plant cell

enlargement, division, and enlargement in symbiotic roots and nonsymbiotic roots as well. Phosphate-solubilizing *B. subtilis* strains have been reported to synergistically increase plant nitrogen and phosphate accumulation when co-inoculated with mycorrhiza *Glomus intraradices* (Toro et al., 1997).

In addition, enhancement of plant growth by root-colonizing *B. amyloliquefaciens* FZB strains is well documented. Researchers showed that bio-fertilization exerted by extracellular bacterial phytase under conditions of phosphate limitation and in the presence of phytate can contribute to the plant-growth-promoting activity of *B. amyloliquefaciens* FZB strains (Idris et al., 2002; Makarewicz et al., 2006). Phytases are enzymes that sequentially remove phosphate groups from myo-inositol 1,2,3,4,5,6-hexakisphosphate (phytate), the main storage form of phosphate in plants. Besides their ability to make phytate phosphorus available, elimination of chelate-forming phytate, which is known to bind nutritionally important minerals (Zn^{2+} , Fe^{2+} , and Ca^{2+}), is another beneficial effect of extracellular phytase activities of *Bacillus* spp. (Kerovuo et al., 1998). Phytase activities of bacteria inhabiting the plant rhizosphere may contribute to their plant growth-promoting effect (Idris et al., 2002).

Makarewicz et al. (2006) identified the *phy C* (phytase) gene from environmental *B. amyloliquefaciens* FZB45 as a member of the phosphate starvation-inducible PhoPR regulon. *In vivo* and *in vitro* assays revealed that PhoP~P is essential for *phy C* transcription. The expression of FZB45 *phy C* is controlled by the PhoPR two-component system. Generally, the PhoPR signal transduction system is induced under phosphate starvation and controls several reactions that increase the cellular supply of soil-living microorganisms with the limiting nutrient phosphate including liberation of phosphate groups from myo-inositol hexakisphosphate by phytase.

Suppression of the competitive plant-pathogenic microflora within the rhizosphere by secreted antifungal and antibacterial lipopeptides and polyketides might be important for promotion of plant growth by FZB42. Koumoutsis et al. (2004) reported that *B. amyloliquefaciens* FZB42 is a producer of three families of lipopeptides, surfactins, bacillomycin D, and fengycins, which are well known secondary metabolites with antifungal activity. Bacillomycin D has been shown to exert the main antifungal activity. Bacillomycin D belongs to the iturin family of lipopeptides, with members such as iturin A and mycosubtilin. It is acyclic heptapeptide with an amino fatty acid moiety and is synthesized

nonribosomally according to the multicarrier thio-template mechanism.

The *bmy* operon (37.2 kb) directs the biosynthesis of bacillomycin D and consists of four genes (*bmy* D, *bmy* A, *bmy* B, and *bmy* C). Synthesis of bacillomycin D is regulated in multiple layers. Expression of the *bmy* operon is dependent on a single σ^A -dependent promoter, P_{bmy} and is favoured in its natural host by the small regulatory protein Deg Q. The global regulator Deg U and Com A are required for the full transcriptional activation of *bmy*. In addition, a transmembrane protein of unknown function, Ycz E, acts on a later level of gene expression and exert posttranscriptional effects with Deg U (Koumoutsis et al., 2007).

Furthermore, three giant gene clusters containing genes with homology to polyketide synthase (PKS) genes of modular organization were identified but not assigned functional roles. Mutants of FZB42 deficient in the synthesis of cyclic-lipopeptides were unable to suppress phytopathogenic fungi but still retained their antibacterial potency. Chen et al. (2006) characterize the giant modular PKS gene clusters *pkc1* and *pkc3*, which are responsible for the biosynthesis of the antibiotics bacillaene and difficidin or oxydifficidin in *B. amyloliquefaciens*.

Recently, Idris et al. (2007) reported that *B. amyloliquefaciens* strain FZB42 able to produce substances with auxin (indole-3-acetic acid (IAA))-like bioactivity. The presence of IAA-like compounds in the culture filtrates of FZB42 was detected by enzyme-linked immunosorbent assay tests with IAA-specific antibodies, when strain was grown at low temperature and low aeration. Using a combined approach of chemical and genetic analysis, biosynthesis of IAA in the PGPR *B. amyloliquefaciens* FZB42 affects its ability to promote plant growth. Moreover, this ability is dependent on the presence of tryptophan, which is one of the main compounds present in several plant exudates (Kamilova et al., 2006). Inactivation of genes involved in tryptophan biosynthesis and in a putative tryptophan-dependent IAA biosynthesis pathway lead to reduction of both IAA concentration and plant growth-promoting activity in the respective mutant strains (Idris et al., 2007).

Colonization of the phytosphere is required for a microbe to directly influence plant health wherein most studies of inoculant strains include analyses of colonization and survival. Most important factors derived from seed that set the stage for early colonization of germinating seedlings by microbes and they have been shown to be important determinants for root colonization by *B. subtilis* GB03 and *B. cereus* (McKellar and Nelson, 2003).

ISR by *Bacillus* spp. and its mechanism

Studies on mechanisms of ISR are suggested to be valuable in extension of PGPR-elicited ISR to practical agriculture. It has been suggested that mixtures of PGPR strains with different mechanisms of interactions might more reliably benefit plants than would individual PGPR strains (Raupach and Kloepper, 1998). Choudhary et al. (2007) elaborately described induced resistance and its mechanism of action in plants. Plants have the ability to acquire enhanced level of resistance to pathogens after exposure to biotic stimuli provided by many different PGPRs. These in association with plant roots elicit a steady state of defense or ISR in plants. This is often referred to as rhizobacteria-mediated ISR. PGPR-elicited ISR was initially observed in carnation, common bean and in cucumber with reduced susceptibility to *Fusarium* wilt, halo blight, and *Colletotrichum orbiculare*, respectively. Several PGPR that colonize root systems with seed applications protect plant against foliar disease include *Pseudomonas fluorescens*, *P. putida*, *B. pumilus*, and *Serratia marcescens* (Thomma et al., 2001).

Induced resistance: general characteristics

Induced resistance is a physiological “state of enhanced defensive capacity” elicited by specific environmental stimuli, whereby the plant’s innate defenses are potentiated against subsequent biotic challenges. This enhanced state of resistance effective against a broad range of pathogens and parasites (van Loon, 2000). Besides ISR, there is another defined form of induced resistance so-called systemic acquired resistance (SAR), which can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved. SAR can be triggered by exposing the plant to virulent, avirulent, and non-pathogenic microbes. Depending on the plant and elicitors, a set period of time is required for the establishment of SAR wherein accumulation of pathogenesis-related proteins (chitinase and glucanase), and salicylic acid (SA) takes place. Unlike SAR, ISR does not involve the accumulation of pathogenesis-related proteins or salicylic acid, but instead, relies on pathways regulated by jasmonate and ethylene (Yan et al., 2002). A network of interconnected signaling pathways regulates induced defenses of plants against pathogens. The primary components of the network are plant signal molecules – SA, jasmonic acid (JA), ethylene (ET), and probably nitric oxide (NO).

SA signaling in plant defense

Extensive studies have shown that SA plays a central role in plant defense against pathogens. SA levels increase in both infected and distal leaves in response to pathogen attack, and exogenous application of SA can induce a set of pathogenesis-related (PR) genes and establish SAR (Uknes et al., 1992). Despite the presence of effective defense systems, many bacterial pathogens are able to evade or suppress the host defense or modulate the metabolism of the host to obtain nutrients for their colonization through virulence strategies. For example, Gram-negative bacteria, such as *P. syringae*, secrete virulence proteins, called effectors, directly into the host cell via a type III secretion system to promote pathogenicity (Mudgett, 2005).

Previous studies have shown that the levels of IAA, the primary plant auxin, increased in plant tissues infected by *P. syringae* pv. tomato (*Pst*) DC3000. Moreover, the type III effector AvrRpt2 modulates host IAA levels to promote pathogen virulence and disease development in *Arabidopsis* (Chen et al., 2004). Recent micro array analysis has shown that infection with *Pst* DC3000 activates genes related to IAA biosynthesis and represses Aux/IAA family and auxin transporter genes, suggesting that *Pst* DC3000 impacts auxin signaling probably through activating IAA production, altering IAA movement, and derepressing the auxin pathway (Thilmony et al., 2006). However, little is known about genes that regulate auxin signaling in response to pathogen infection and the downstream targets of altered auxin signaling responsible for enhanced disease susceptibility. Auxin rapidly induces numerous genes called early auxin response genes. The best characterized early auxin response genes include three major classes: Aux/IAAs, SAURs, and GH3s (Hagen and Guilfoyle, 2002).

Further studies have shown that GH3 genes encode IAA-amido synthetases that are involved in auxin homeostasis through conjugating amino acids to IAA. It was also shown that GH3.5 (At4g27260) adenylated both IAA and SA *in vitro* (Staswick et al., 2005), suggesting that GH3.5 could function in modulating and integrating both auxin and SA signaling in the plant–pathogen interaction. Zhang et al. (2007) investigated the functions of GH3.5 in response to *P. syringae* infection and showed that GH3.5 positively regulates the SA signaling pathway in plant defense and modulates the auxin pathway to enhance host susceptibility. Conclusively they demonstrated that GH3.5 is a key modulator that both positively and negatively affects various aspects of plant defense, revealing another dimen-

sion to the complex and dynamic plant–pathogen interaction.

Role of JA- and ET-signaling in ISR

Signal transduction leading to ISR has been seen to be triggered by several low molecular weight volatile compounds of microbial origin in the rhizosphere that may even include any of the above molecules. It has been suggested that signal transduction leading to ISR requires responsiveness to both JA and ET (Figure 2). Methyl jasmonate (MeJA) and the ET precursor i.e., ACC are effective in inducing resistance against phytopathogenic microflora (Thomma et al., 2001; Yan et al., 2002).

It is postulated that ET signaling is required at the site of application of inducers which are involved in the generation or translocation of the systemically transported ISR signals. Mutation in plant signaling pathways point to an active role by SA or JA and/or ET in activating ISR. JA and ET act in concert in activating defense responses. JA and derivatives induce the expression of genes encoding defense-related proteins e.g., thionins and proteinase inhibitors whereas ET activates several members of the pathogenesis-related (PR) gene super family. They also act synergistically in stimulating elicitor-induced PR gene expression and systematically induce defense responses.

Functional role of NO signaling in plant defense

In addition to SA, JA, and ET, NO, first characterized as an endothelium-derived relaxation factor, is involved in diverse cellular processes including neuronal signaling, blood pressure homeostasis, and immune response. Recent studies have also revealed a role for NO as a signaling molecule in plants. As a developmental regulator, NO promotes germination, leaf extension and root growth, and delays leaf senescence and fruit maturation. Moreover, NO acts as a key signal in plant resistance to incompatible pathogens by triggering resistance-associated hypersensitive cell death. In addition, NO activates the expression of several defense genes (e.g. pathogenesis-related genes, phenylalanine ammonia-lyase, chalcone synthase) and could play a role in pathways leading to systemic acquired resistance (Romero-Puertas and Delledonne, 2003).

The plant resistance response to pathogen attack begins with specific recognition by plant receptors of the invading pathogen. The avirulent pathogen elicits a set of plant defense responses (Figure 3)

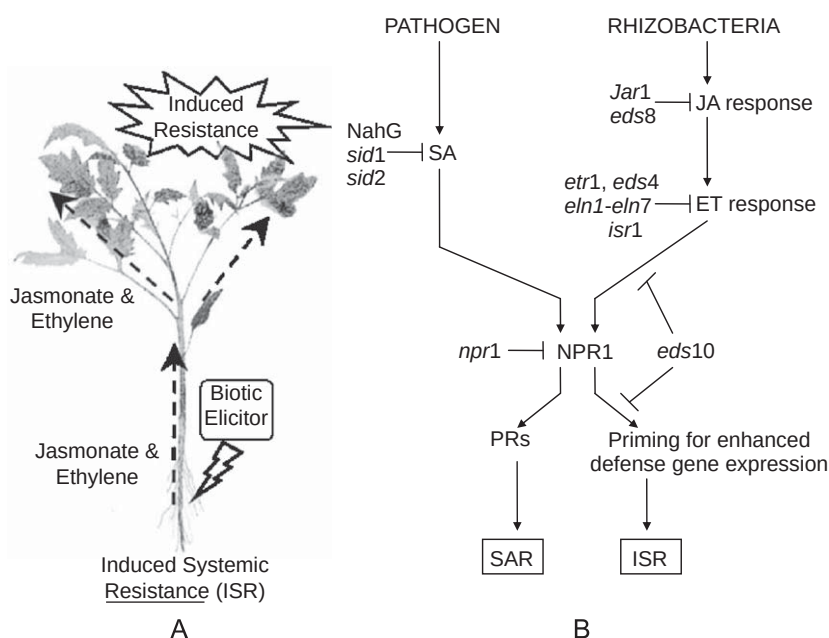


Figure 2. Pictorial representation of induced systemic resistance. (A) Induced by the exposure of roots to specific strains of plant growth-promoting rhizobacteria, is dependent of the phytohormones ethylene and jasmonate (jasmonic acid), and independent of salicylate. (B) The pathogen-induced SAR and the rhizobacteria-mediated ISR signal transduction pathways. Source: Vallad and Goodman (2004).

that usually results in the so-called hypersensitive reaction (HR), which is characterized by collapse of the infected cells. This hypersensitive cell death delimits the infected zone and avoids the multiplication and spread of the pathogen (Heath, 1998). One of the earliest events in the HR is the rapid accumulation of reactive oxygen species (ROS) and NO through the activation of enzyme systems similar to neutrophil NADPH oxidase (Keller et al., 1998) and nitric oxide synthase (NOS) (Chandok et al., 2003). Both NO and ROS are necessary to trigger host cell death and are also components of a highly amplified and integrated defense system that involves SA, activation of ion fluxes, changes in protein phosphorylation patterns, extracellular pH, membrane potential, oxidative cross-linking of plant cell wall proteins, and perturbations in the level of cytosolic Ca^{2+} (Shirasu et al., 1997).

NO function together with ROS in triggering hypersensitive cell death, but is also involved in other defense functions complementary to, and independent of, ROS. There is evidence that NO stimulates a signal transduction pathway for the production of phytoalexin in higher plant cells (Noritake et al., 1996). Moreover, the first enzyme of phenylpropanoid biosynthesis pathway, phenylalanine ammonia-lyase (PAL), is induced after infiltration of tobacco leaves and cells with commercially available NOS or NO donors (Durner et al., 1998) and inhibition of NOS activity clearly

reduces its transcript accumulation (Delledonne et al., 1998). It has also been shown that the response of soybean cotyledons to elicitors from *Diaporthe phaseolorum* f. sp. *meridionalis* triggers the biosynthesis of antimicrobial flavonoids via NOS-like activity. Furthermore, the use of NOS inhibitors caused a marked reduction in the expression of chalcone synthase (CHS), the first enzyme of the branch specific for flavonoids and isoflavonoid-derived antibiotics (Dixon and Paiva, 1995).

As accumulation of PAL and PR1, a pathogenesis-related protein, has been detected in tobacco cell suspensions treated with a membrane permeable analogue of cGMP, and taking into account that induction of PAL in tobacco suspension cells by NO can be suppressed by several inhibitors of guanylate cyclase, activation of a NO-dependent defense gene is thought to be mediated by cGMP (Durner et al., 1998). However, PAL expression is not fully blocked by inhibitors of cGMP production, implying that different modes of PAL induction operated own stream of NO.

Cyclic ADP ribose (cADPR) has been implicated as another second messenger for NO signaling in animals, acting in a cGMP-dependent signaling cascade to mediate calcium mobilization. Treatment with cADPR induces PAL and PR-1 expression in tobacco, which can be inhibited by ruthenium red, indicating its calcium dependence. Moreover, a cADPR antagonist suppressed NO induction of

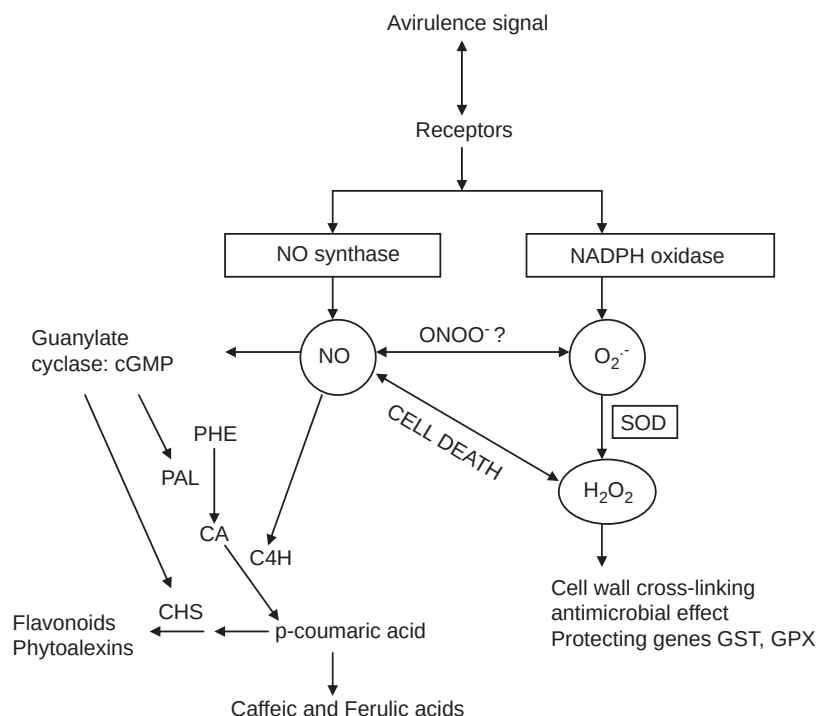


Figure 3. NO- and H₂O₂-mediated plant hypersensitive disease resistance response. Ca²⁺, Ca²⁺ influx; CA, cinnamic; CHS, chalcone synthase; GPX, glutathione peroxidase; GST, glutathione S-transferases; P, phosphorylation dependent step, ONOO⁻ peroxynitrite; PAL, phenylalanine ammonia-lyase; PHE, phenylalanine; SOD, superoxide dismutase. Source: Romero-Puertas and Delledonne (2003).

PR-1; however, this effect was incomplete, indicating that NO activation of defense responses may occur through more than one pathway (Durner et al., 1998).

Both positive and negative regulation of plant defense responses operating downstream of the generation of NO and ROS have also been attributed to mitogen-activated protein kinase (MAPK) cascades. A MAPK was recently found to be activated by NO in *Arabidopsis* (Clarke et al., 2000).

Plants respond to bacterial QS signals

In addition, bacterial infection of plants often depends on the exchange of QS signals between nearby bacterial cells. It is now evident that plants, in turn, 'listen' to these bacterial signals and respond in sophisticated ways to the information (Bauer and Mathesius, 2004). The first global studies of eukaryotic responses to AHLs were done on the model plant *Medicago truncatula*. When roots were exposed to physiological levels of AHLs (1–100 nM), the levels of over 100 of the 2000 resolvable root proteins changed by fourfold or more. AHLs affect a wide range of functions in *M. truncatula*, including defense and stress responses, transcriptional regulation, protein proces-

sing, responses to plant hormones, and cytoskeletal elements as well as primary and secondary metabolism (Figure 4). The systemic transmission of responses to AHL-QS signals throughout the organisms could be an important aspect of integrating a plant's overall preparation for dealing with bacterial assault or the establishment of a mutualistic relationship (Bauer et al., 2005).

N-acyl-L-homoserine lactone (AHL) signal molecules are utilized by Gram-negative bacteria to monitor their population density (QS) and to regulate gene expression in a density-dependent manner. Schuëgger et al. (2006) showed that *Serratia liquefaciens* MG1 and *Pseudomonas putida* IsoF colonize tomato roots, produce AHL in the rhizosphere and increase systemic resistance of tomato plants against the fungal leaf pathogen, *Alternaria alternata*. The AHL-negative mutant *S. liquefaciens* MG44 was less effective in reducing symptoms and *A. alternata* growth as compared to the wild type. SA levels were increased in leaves when AHL-producing bacteria colonized the rhizosphere. No effects were observed when isogenic AHL-negative mutant derivatives were used in these experiments. Furthermore, macroarray and Northern blot analysis revealed that AHL molecules systemically induce SA- and ET-dependent defense genes (i.e. PR1a, 26 kDa acidic and 30 kDa basic

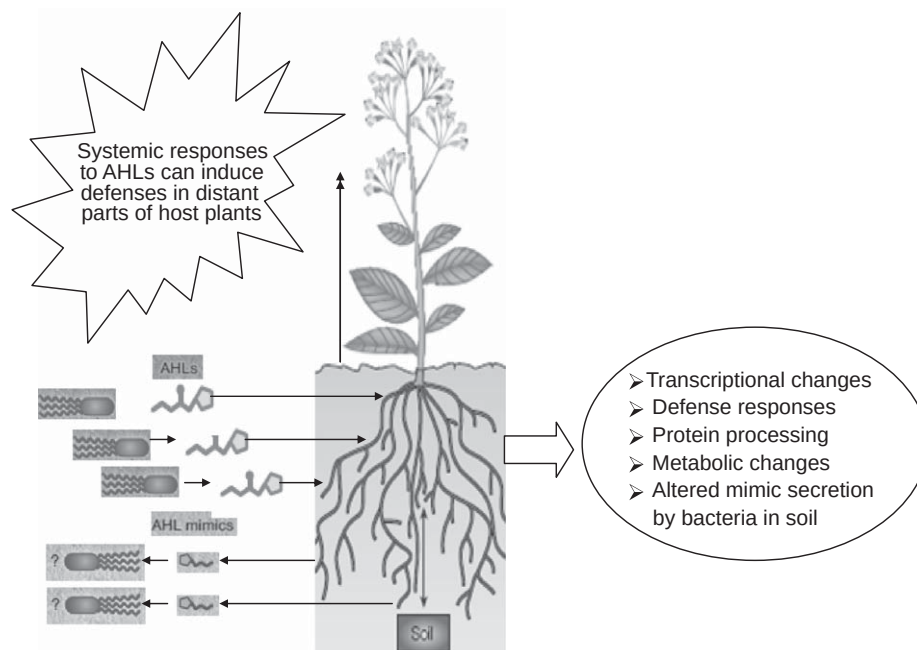


Figure 4. A eukaryotic host listens to bacterial AHL conversations. AHLs produced by associated bacteria are detected by both the host and the bacteria. The host responds by altering many functions, including defenses, metabolism and production of AHL mimic compounds. Systemic responses to the bacterial AHLs can induce defenses in distant parts of the host.

chitinase). Together, these data support the view that AHL molecules play a role in the biocontrol activity of rhizobacteria through the induction of systemic resistance to pathogens.

Rhizobacteria-induced systemic resistance in plants

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 *Arabidopsis thaliana* (van Loon and Glick, 2004). Fewer published accounts of ISR by *Bacillus* spp. are available which showed 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 (Table 3) 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 (green kuang futsoi and long cayenne pepper) (Kloepper et al., 2004a).

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 was associated with enhanced peroxidase activity and increased production of one chitinase isozyme and two isozymes of β -1,3-glucanase that produced by *B. mycoides* strain Bac J (Bargabus et al., 2002), and *B. pumilus* strains 203-6 and 203-7 (Bargabus et al., 2004), respectively. In the tobacco blue mold system, Zhang et al. (2002) reported that plants treated with *B. pumilus* strain SE34 had greatly increased levels of SA, 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. (1996, 1998) with *B. pumilus* strain SE34. In the first study (Benhamou et al., 1996), 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

Table 3. Examples of *Bacillus*-based biocontrol in plants against pathogens

Plant	Disease	Pathogen	Biocontrol <i>Bacillus</i>	Reference
Sugarbeet	Cercospora leaf spot	<i>Cercospora beticola</i>	<i>B. pumilus</i> strains 203-6 & 203-7, <i>B. mycoides</i> strain Bac J	Bargabus et al. (2002, 2004)
Radish	Bacterial leaf spot	<i>Xanthomonas campestris</i> pv. <i>armoraciae</i>	<i>Bacillus</i> spp.	Krause et al. (2003)
Cucumber	Root rot	<i>Pythium</i> spp.	<i>Bacillus</i> spp.	Zhang et al. (1996)
Peanut	Late leaf spot	<i>Cercosporidium personatum</i>	<i>Bacillus</i> spp.	Zhang et al. (2001)
Tobacco	Blue mold	<i>Peronospora tabacina</i>	<i>B. pasteurii</i> C-9, <i>B. pumilus</i> SE34 and T4	Zhang et al. (2002, 2004)
Cucumber	Bacterial wilt	<i>Erwinia tracheiphila</i>	<i>B. pumilus</i> INR7	Zehnder et al. (1997)
Tomato	Leaf spot	Cucumber mosaic virus (CMV)	<i>B. subtilis</i> IN937b, <i>B. pumilus</i> SE34, <i>B. amyloliquefaciens</i> IN937a	Zehnder et al. (2000)
Tomato	Late blight	<i>Phytophthora infestans</i>	<i>B. pumilus</i> SE34	Yan et al. (2003)
Loblolly pine	Fusiform rust	<i>Cronartium quercuum</i> , <i>Miyabe exsirai</i> f. sp. <i>fusiforme</i>	<i>B. sphaericus</i> SE56 and <i>B. pumilus</i> INR7, SE34, SE49, SE52	Enebak et al. (1998)
Peanut	Crown rot	<i>Aspergillus niger</i>	<i>B. subtilis</i> AF1	Sailaja et al. (1997)
Cucumber	Angular leaf spot	<i>P. syringae</i>	<i>B. pumilus</i> INR7	Wei et al. (1996)
Tomato	Anthraxnose	<i>Colletotrichum arbutum</i>	<i>B. amyloliquefaciens</i> IN937a, <i>B. subtilis</i> IN937b, and <i>B. pumilus</i> SE34	Murphy et al. (2000)
Tomato	Leaf spot	Tomato mottle virus (ToMoV)	<i>B. pumilus</i> strain T4	Park and Kloepper (2000)
Tobacco	Wild fire	<i>P. syringae</i> pv. <i>tabaci</i>	<i>Bacillus</i> spp.	Jetiyanon and Kloepper (2002)
Tomato	Bacterial wilt	<i>Ralstonia solanacearum</i>		
Long cayenne pepper	Anthraxnose	<i>Colletotrichum gloeosporioides</i> (Penz.) Penz and Sacc.		
Green kuang	Damping-off	<i>Rhizoctonia solani</i>		
futsoi				
Cucumber	Leaf spot	CMV		

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 (Benhamou et al., 1998), 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 tomato late blight system reported by Yan et al. (2002), 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 break down endogenous SA, but not in the ET-insensitive NR/NR line or in the JA insensitive *df1/df1* line. These results are consistent with studies on several strains of *Pseudomonas* spp. that elicit ISR in *Arabidopsis thaliana* (van Loon and Glick, 2004) where ISR is typically independent of SA 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. (2002). 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 SA-independent pathways confirms to the model with ISR elicited by *Pseudomonas* spp. in *A. thaliana* (van Loon and Glick, 2004).

Different results were found with *B. pumilus* strain T4 (Park and Kloepper, 2000) that elicited ISR in tobacco against wildfire, caused by *Pseudomonas syringae* pv. *tabaci*. In this system, a bacterized 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 SA-dependent signaling pathway (van Loon and Glick, 2004). In another study of signaling pathways, Ryu et al. (2003) found different results with strain T4 in *Arabidopsis* spp. In this study, *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 whereas strains IN937a and GB03 did not elicit protection against either pathovar. 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 an SA-independent pathway was dominant in the tests, an SA-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. (Ryu et al., 2003) revealed that in agreement with results on signaling during ISR elicited by *Pseudomonas* spp., ISR elicited by strain SE34 was dependent on NPR1, JA, and ET, while ISR elicited by strain T4 was dependent on ET. In contrast to results on signaling during ISR elicited by *Pseudomonas* spp., ISR elicited by strain T4 was independent of NPR1 and JA.

In addition, Ryu et al. (2003) examine whether volatile organic compounds associated with rhizobacteria are involved in the activation of ISR in *Arabidopsis*. They report that the exposure of *Arabidopsis* seedlings to bacterial volatile blends from strains of *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a significantly reduced the severity of disease caused by the bacterial pathogen *Erwinia carotovora*. Chemical analysis of the bacterial volatile emissions revealed the presence of a series of low- M_r hydrocarbons including the growth-promoting chemical 2R, 3R-butanediol. The exogenous application of a racemic mixture of 2,3-butanediol was also found to trigger ISR in *Arabidopsis*. Transgenic lines of *B. subtilis* that emitted lower levels of 2,3-butanediol were less effective than wild type in protecting *Arabidopsis* against pathogen infection. Using transgenic and mutant lines of *Arabidopsis*, the authors provide evidence that the signaling pathway activated by volatiles is dependent on ET, and independent of the SA or JA signaling pathways.

Collectively, the results reported to date on signaling pathways of ISR elicited by *Bacillus* spp. demonstrate the following points. Several 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.

Role of volatiles in ISR that produced by *Bacillus*

The role of volatiles of microbial origin as signal molecules for plant defense has come to light

recently. A comparison has been drawn between herbivores-induced plant volatiles (HIPVs) as an elicitor of plant defenses and two other classes of signaling molecules, C6 green-leaf volatiles (GLVs) and C4 bacterial volatiles which appear to prime plant defenses thereby enhancing the capacity to mobilize cellular defense responses when plants are faced with herbivore/pathogen attacks (Choudhary et al., 2008). Volatile signals generated by certain non-pathogenic bacteria have also been shown to trigger defense responses in *Arabidopsis* (Ryu et al., 2003).

Ryu et al. (2004) examined the role of airborne bacterial metabolites in triggering ISR by growing PGPR and *Arabidopsis* seedlings on separate sides of divided Petri dishes. ISR was activated by exposure of *Arabidopsis* seedlings to volatile organic compounds (VOCs) from the *Bacillus* sp. on continuous exposure for as short as 4 days by a significant reduction in symptomatic leaves inoculated with the soft rot-causing pathogen *Erwinia carotovora*. VOCs collected from growth-promoting bacteria *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a, showed consistent difference in the composition of volatile blends compared to VOCs, which were recovered from non growth-promoting bacterial strain DH5 α . Strains GB03 and IN937a consistently released two most abundant compounds, 2,3-butanediol and 3-hydroxy-2-butanone (acetoin) while these metabolites were not released from DH5 α or water treated MS media (Ryu et al., 2003).

Several other VOCs were also observed which included dodecane, 2-undecanone, 2-tridecanone, 2-tridecanol and tetramethyl pyrazine from complex bacterial bouquet that did not exhibit ISR priming activity. Bacteria employ different mechanisms to produce VOCs e.g., in *Bacillus* sp. strain GB03 and IN937a, 2,3-butanediol and acetoin were produced under low atmospheric O₂ partial pressure to provide an alternative electron sink for the regeneration of NAD⁺ when usual respiration was not possible (Ryu et al., 2004).

No disease protection was observed when *Bacillus* strains were genetically blocked for the production of 2,3-butanediol; this confirmed the priming activity of the compound to induce resistance against disease. The involvement of known signaling pathways in *Arabidopsis* was screened by exposing defined mutants and transgenic plant lines to bacterial emissions containing VOCs especially 2,3-butanediol. ISR triggered by GB03 VOC was independent of SA, NPR1, and JA signaling pathway but was more or less mediated by ET. Interestingly, ISR activation by strain IN937a was independent of all the signaling pathways and this opens up the possibility of involvement of addi-

tional VOCs which utilize alternative pathways to trigger ISR.

Ryu et al. (2004) conducted Petri dish assays wherein they exposed the whole plant to the plume of bacterial VOCs, which reflect as to whether the site of plant VOC perception is above or below-ground for soil-grown plants. The sphere of microbial VOCs for rhizobacteria could be within the soil or above ground and the possibility existed that VOCs are produced at sufficient level for aerial tissues to perceive and respond to bacterial volatiles. Alternatively, an endogenous signal or signal transports information from the root zone to the aerial portion of the plant and this necessitates the presence of some mobile messenger within the plant because of systemic nature of induced resistance.

Collectively, the result of studies on VOCs (Podile and Dube, 1988; Park and Kloepper, 2000) demonstrate that (i) whole VOCs from two different *Bacillus* spp., viz., *B. subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a, 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 (ET-dependent).

The biofertilizing effect of *Bacillus* spp. in conjunction with ISR

It is sure that *Bacillus* spp. will be used more in production agriculture and horticulture based on recent progresses shown in implementing microbial inoculants. 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 (Table 4). As more such studies are done for specific crops in various geographical areas, adoption of microbial inoculants will most likely increase (Kloepper et al., 2004b).

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. 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 (Table 4). Yield Shield consists of a spore preparation of the *B. pumilus* strain INR7 and designated by Gustafson, LLC as GB34. 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. Another way to overcome the problem of variability from using microbial inoculants alone for disease control would be to combine the use of microbial treatments with suboptimal concentrations of fungicides.

Bargabus et al. (2002) showed 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. 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 (Raupach and Kloepper, 1998). Jetiyanon and Kloepper (2002) extended a previous greenhouse study using mixtures of *Bacillus* strains that elicit ISR 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 (Kloepper et al., 2004b). 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

Table 4. Commercially available *Bacillus*-based plant disease biocontrol products

Product name	Company	<i>Bacillus</i> component	Formulation type ^a	Primary target
Serenade	AgraQuest, Davis, CA	<i>B. subtilis</i> QST 713	WP, aqueous. Suspension	Fungi, bacteria on multiple fruits
EcoGuard	Novozymes, Salem, VA	<i>B. licheniformis</i> SB3086	Flowable	<i>Sclerotinia homoeocarpa</i> on turf
Kodiak	Gustafson, Plano, TX	<i>B. subtilis</i> GB03	WP, Flowable	Fungi on cotton, legumes, soybeans
Yield Shield	Gustafson	<i>B. pumilus</i> GB34	WP	Fungi on soybeans
BioYield	Gustafson	<i>B. amyloliquefaciens</i> GB99+ <i>B. subtilis</i> GB122	Dry flake	Fungi on bedding plants in potting mixes
Subtilex	Beker Underwood, Ames, IA	<i>B. subtilis</i> MB1600	WP	Fungi on cotton, legumes, soybeans
Hi Stick L+Subtilex	Beker Underwood	<i>B. subtilis</i> MB1600+ <i>Rhizobium</i>	Flowable	Fungi on soybeans, peanut

Source: Schisler et al. (2004).

^aWP = wettable powder.

were chitosan for nematode control via promotion of indigenous soil predators and antagonists to root-knot nematodes, *B. subtilis* strain GB03 for control of soil-borne 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 (Kloepper et al., 2004b). 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 (Kloepper et al., 2004b). 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 and future perspectives

This review has focused on recent research concerning interactions between biocontrol agents and pathogens in the phytosphere. Greatest interest recently has concerned the phenomenon of induced resistance as the molecular tools for its study became available and more work in this area is clearly justified. Multiple populations of *Bacillus* can contribute to plant health in a variety of ways. Numerous isolates of this genus have been developed as biocontrol agents of plant pests and pathogens. 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. Second, the same strains of *Bacillus* spp. that elicit ISR typically promote plant growth. 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.

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. Most microbes produce and secrete

one or more secondary metabolites with antibiotic activity. In some instance antibiotics produced by microorganisms have been shown to be particularly effective at suppressing plant pathogens and the diseases they cause. To be effective, antibiotics must be produced in sufficient quantities near the pathogen to result in a biocontrol effect. *In situ* production of antibiotics by several different biocontrol agents has been measured (Thomashow et al., 2002); however, the effective quantities are difficult to estimate because of the small quantities produced relative to the other, less toxic, organic compounds present in the phytosphere. Several biocontrol strains are known to produce multiple antibiotics which can suppress one or more pathogens. For example, *B. cereus* strain UW85 is known to produce antibiotics zwittermycin (Silo-Suh et al., 1994) and kanosamine (Milner et al., 1996). The ability to produce multiple classes of antibiotics by *Bacillus* spp., probably helps to suppress diverse microbial competitors, some of which are likely to be phytopathogens. 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.

In order to more successfully apply such agents, a greater understanding of their ecology is needed. Indeed, the safety and efficacy of inoculants will be determined in a large part by the ecological success of the applied strains in the environments into which they are introduced. Greater knowledge of the diversity, distribution, and activities of *Bacillus* spp. will be useful for identification of new inoculant strains and the cropping systems into which they can be most profitably applied.

From a more general perspective, the diversity within populations of antagonistic microorganisms with a common biocontrol trait is a means to improving biocontrol. This approach builds on existing knowledge of mechanisms while exploiting genetic differences that have evolved to enable microbial populations to compete successfully in diverse soil and rhizosphere environments. Understanding the diversity within populations of biocontrol agents holds the promise of pairing specific genotypes with their most supportive plant hosts or soil environments to maximize root colonization and disease suppression. In addition, agricultural management practices as well as the “history” of cultivation in a crop rotation cycle may be supportive or contra-productive for the successful establishment of biocontrol active Bacilli in a given crop.

Finally, establishing the presence and functionality of individual populations within a particular

soil is just one first step toward fully understanding the nature of suppressiveness within that soil. Ultimately, the parameters within which the activities of functionally important microbial populations combine to produce a suppressive soil also must be defined. To identify those parameters, new and more detailed studies will be required to characterize the soil structure and composition, the environmental conditions under which suppression occurs, the molecular interactions among functionally important populations under different conditions, and the biogeography and population dynamics of beneficial as well as pathogenic microbial populations in the field. Because of the complexity of field soils, high throughput methods will be required to adequately characterize these populations, but the pay-off will be worth the effort. The future studies of biologically based soil suppressiveness will present new insights in to the microbial ecology of agricultural soils and lay the foundation for the development of creative management strategies for the suppression of soil-borne diseases.

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References

- Arias RS, Sagardoy MA, van Vuurde JWL. Spatio-temporal distribution of naturally occurring *Bacillus* spp. and other bacteria on the phylloplane of soybean under field conditions. *J Basic Microbiol* 1999;39:283–92.
- Audenaert K, Pattery T, Comelis P, Hötte M. Induction of systemic resistance to *Botrytis cinerea* in tomato by *P. aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. *Mol Plant Microbe Interact* 2002;15:1147–56.
- Bargabus RL, Zidack NK, Sherwood JW, Jacobsen B J. Characterization of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. *Physiol Mol Plant Pathol* 2002;61:289–98.
- Bargabus RL, Zidack NK, Sherwood JW, Jacobsen BJ. Screening for the identification of potential biological control agents that induce systemic acquired resistance in sugar beet. *Biol Control* 2004;30:342–50.
- Bauer WD, Mathesius U. Plant responses to bacterial quorum sensing signals. *Curr Opin Plant Biol* 2004;7:429–33.
- Bauer WD, Mathesius U, Teplitski M. Eukaryotes deal with bacterial quorum sensing. *ASM News* 2005;71:129–35.
- Benhamou N, Kloepper JW, Quadri-Hallmann A, Tuzun S. Induction of defense-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. *Physiol Plant Pathol* 1996;112:919–29.
- Benhamou N, Kloepper JW, Tuzun S. Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of the host response. *Planta* 1998;204:153–68.
- Chandok MR, Ytterberg AJ, van Wijk KJ, Klessig DF. The pathogen-inducible nitric oxide synthase (iNOS) in plants is a variant of the P protein of the glycine decarboxylase complex. *Cell* 2003;113:469–82.
- Chen X-H, Vater J, Piel J, Franke P, Scholz R, Schneider K, et al. Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. *J Bacteriol* 2006;188:4024–36.
- Chen X-H, Koumoutsis A, Scholz R, Eisenreich A, Schneider K, Schneider I, et al. Comparative analysis of the complete genome sequence of the plant growth promoting *Bacillus amyloliquefaciens* FZB42. *Nat Biotechnol* 2007;25:1007–14.
- Chen Z, Kloeck AP, Cuzick A, Moeder W, Tang D, Innes RW, et al. The *Pseudomonas syringae* type III effector AvrRpt2 functions downstream or independently of SA to promote virulence on *Arabidopsis thaliana*. *Plant J* 2004;37:494–504.
- Choudhary DK, Prakash A, Johri BN. Induced systemic resistance (ISR) in plants: mechanism of action. *Indian J Microbiol* 2007;47:289–97.
- Choudhary DK, Johri BN, Prakash A. Volatiles as priming agent that initiate plant growth and defense responses. *Curr Sci* 2008;94:595–604.
- Clarke A, Desikan R, Hurst RD, Hancock JT, Neill SJ. NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant J* 2000;24:667–77.
- De Meyer G, Capieau K, Audenaert K, Buchela A, Métraux JP, Höfte M. Nanogram amounts of SA produced by rhizobacteria *P. aeruginosa* 7NSK2 activate the SAR pathway in bean. *Mol Plant-Microbe Interact* 1999a;12:450–8.
- De Meyer G, Audenaert K, Höfte M. *Pseudomonas aeruginosa* 7NSK2-induced systemic resistance in tobacco depends on in planta salicylic acid accumulation but is not associated with PR1a expression. *Eur J Plant Pathol* 1999b;105:513–7.
- Delledonne M, Xia Y, Dixon RA, Lamb C. Nitric oxide functions as a signal in plant disease resistance. *Nature* 1998;394:585–8.
- Dixon RA, Paiva N. Stress-induced phenyl propanoid metabolism. *Plant Cell* 1995;7:1085–97.
- Durner J, Wendehenne D, Klessig DF. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc Natl Acad Sci USA* 1998;95:10328–33.

- Enebak SA, Wei G, Kloepper JW. Effects of plant growth-promoting rhizobacteria on loblolly and slash pine seedlings. *For Sci* 1998;44:139–44.
- Felske ADM, Heyrman J, Balcaen A, de Vos P. Multiplex PCR screening of soil isolates for novel *Bacillus*-related lineages. *J Microbiol Methods* 2003;55:447–58.
- Fray RG. Altering plant-microbe interaction through artificially manipulating bacterial quorum sensing. *Ann Bot* 2002;89:245–53.
- Hagen G, Guilfoyle T. Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Mol Biol* 2002;49:373–85.
- Heath MC. Apoptosis, programmed cell death and the hypersensitive response. *Eur J Plant Pathol* 1998;104:117–24.
- Iavicoli A, Boutet E, Buchela A, Métraux J-P. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *P. fluorescens* CHA0. *Mol Plant Microbe Interact* 2003;16:851–8.
- Idris EE, Makarewicz O, Farouk A, Rosner K, Greiner R, Bochow H, et al. Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth promoting effect. *Microbiology* 2002;148:2097–109.
- Idris EES, Iglesias DJ, Talon M, Borriss R. Tryptophane-dependent production of indole-3-acetic acid (IAA) affects level of plant growth-promotion by *Bacillus amyloliquefaciens* FZB42. *Mol Plant-Microbe Interact* 2007;20:619–26.
- Jetiyanon K, Kloepper JW. Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biol Control* 2002;24:285–91.
- Johri BN, Sharma A, Virdi JS. Rhizobacterial diversity in India and its influence on soil and plant health. *Adv Biochem Engg/Biotechnol* 2003;84:49–89.
- Kamilova F, Kravchenko LV, Shaposhnikov AI, Azarova T, Makarova N, Lugtenberg B. Organic acids, sugars, and L-tryptophan in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. *Mol Plant-Microbe Interact* 2006;19:250–6.
- Keller T, Damude HG, Werner D, Doerner P, Dixon RA, Lamb C. A plant homolog of the neutrophil NADPH oxidase gp91^{phox} subunit gene encodes a plasma membrane protein with Ca²⁺ binding motifs. *Plant Cell* 1998;10:255–66.
- Kerovuo J, Lauraeus M, Nurminen P, Kalkkinen N, Apajalahti J. Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. *Appl Environ Microbiol* 1998;64:2079–85.
- Kloepper JW, Ryu C-M, Zhang S. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 2004a;94:1259–66.
- Kloepper JW, Reddy MS, Kenney DS, Vavrina C, Kokalis-Burelle N, Martinez-Ochoa, N., 2004b. Theory and applications of rhizobacteria for transplant production and yield enhancement. In: Nicola S, Nowak J, Vavrina CS, editors. *Proceedings of the XXVI IHC – transplant production and stand establishment*. Acta Hort 631, 217–29.
- Koumoutsis A, Chen X-H, Henne A, Liesegang H, Hitzeroth G, Franke P, et al. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. *J Bacteriol* 2004;186:1084–96.
- Koumoutsis A, Chen X-H, Vater J, Borriss R. DegU and YczE positively regulate the synthesis of Bacillomycin D by *Bacillus amyloliquefaciens* FZB42. *Appl Environ Microbiol* 2007;73:6953–64.
- Krause MS, De Ceuster TJJ, Tiquia SM, Michel FC, Madden LV, Hoitink HAJ. Isolation and characterization of rhizobacteria from composts that suppress the severity of bacterial leaf spot of radish. *Phytopathology* 2003;93:1292–300.
- Leeman M, Den OFM, van Pelt JA, Dirkx FPM, Steijl H, Bakker PAHM, et al. Iron availability affects induction of SR against Fusarium wilt of radish by *P. fluorescens*. *Phytopathology* 1996;86:149–55.
- Makarewicz O, Dubrac S, Msadek T, Borriss R. Dual role of the PhoP~P response regulator: *Bacillus amyloliquefaciens* FZB45 phytase gene transcription is directed by positive and negative interaction with the *phy* C promoter. *J Bacteriol* 2006;188:6953–65.
- Maurhofer M, Hase C, Meuwly P, Métraux JP, Défago G. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0: influence of the *gacA* gene and of pyoverdine production. *Phytopathology* 1994;84:139–46.
- McKellar ME, Nelson EB. Compost induced suppression of *Pythium* damping-off is mediated by fatty-acid-metabolizing seed-colonizing microbial communities. *Appl Environ Microbiol* 2003;69:452–60.
- McSpadden Gardener BB. Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. *Phytopathology* 2004;94:1252–8.
- Meziane H, Vander SI, van Loon LC, Höfte M, Bakker PAHM. Determinants of *P. putida* WCS358 involved in induced systemic resistance in plants. *Mol Plant Pathol* 2005;6:177–85.
- Milner JL, Silo-Suh L, Lee JC, He H, Clardy J, Handelsman J. Production of kanosamine by *Bacillus cereus* UW85. *Appl Environ Microbiol* 1996;62:3061–5.
- Mudgett MB. New insights to the function of phytopathogenic bacterial type III effectors in plants. *Annu Rev Plant Biol* 2005;56:509–31.
- Murphy JF, Zehnder GW, Schuster DJ, Sikora EJ, Polston JE, Kloepper JW. Plant growth-promoting rhizobacterial mediated protection in tomato against tomato mottle virus. *Plant Dis* 2000;84:779–84.
- Noritake T, Kawakita K, Doke N. Nitric oxide induces phytoalexin accumulation in potato tuber tissues. *Plant Cell Physiol* 1996;37:113–6.
- Park KS, Kloepper JW. Activation of PR-1a promoter by rhizobacteria that induce systemic resistance in tobacco against *Pseudomonas syringae* pv. *tabaci*. *Biol Control* 2000;18:2–9.

- Pathak A, Sharma A, Johri BN. *Pseudomonas* strain GRP₃ induces systemic resistance to sheath blight in rice. *Interl Rice Res Notes* 2004;29:35–6.
- Pinchuk IV, Bressollier P, Sorokulova IB, Verneuil B, Urdaci MC. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. *Res Microbiol* 2002;153:269–76.
- Podile AR, Dube HC. Plant growth-promoting activity of *Bacillus subtilis* strain AF1. *Curr Sci* 1988;57:183–6.
- Priest F. Systematics and ecology of *Bacillus*. In: *Bacillus subtilis* and other Gram-positive bacteria, biochemistry, physiology, and molecular genetics. Washington: ASM Press; 1993. p. 3–16.
- Raupach GS, Kloepper JW. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* 1998;88:1158–64.
- Romero-Puertas MC, Delledonne M. Nitric oxide signaling in plant–pathogen interactions. *IUBMB Life* 2003;55:579–83.
- Ryan PR, Delhaize E. Function and mechanism of organic anion exudation from plant roots. *Annu Rev Plant Physiol Plant Mol Biol* 2001;52:527–60.
- Ryu C-M, Hu C-H, Reddy MS, Kloepper JW. Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. *New Phytol* 2003;160:413–20.
- Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Kloepper JW, et al. Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol* 2004;134:1017–26.
- Sailaja PR, Podile AR, Reddanna P. Biocontrol strain *Bacillus subtilis* AF1 rapidly induces lipooxygenase in groundnut (*Arachis hypogaea* L.) compared to crown rot pathogen *Aspergillus niger*. *Eur J Plant Pathol* 1997;104:125–32.
- Schisler DA, Slininger PJ, Behle RW, Jackson MA. Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology* 2004;94:1267–71.
- Schuhegger R, Ihring A, Gantner S, Bahnweg G, Knappe C, Vogg G, et al. Induction of systemic resistance in tomato by *N*-acyl-L-homoserine lactone-producing rhizosphere bacteria. *Plant Cell Environ* 2006;29:909–18.
- Sharma A, Johri BN. Combat of iron-deprivation through a plant growth promoting fluorescent *Pseudomonas* strain GRP₃A in mung bean. *Microbiol Res* 2003;158:77–81.
- Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *Plant Cell* 1997;9:261–70.
- Siddiqui IA, Saukat SS. Systemic resistance in tomato induced by biocontrol bacteria against the root-knot nematode, *Meloidogyne javanica* is independent of SA production. *Phytopathology* 2004;152:48–54.
- Silo-Suh LA, Lethbridge BJ, Raffel SJ, He H, Clardy J, Handelsman J. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Appl Environ Microbiol* 1994;60:2023–30.
- Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, et al. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant dependent enrichment and seasonal shifts revealed. *Appl Environ Microbiol* 2001;67:4742–51.
- Stahly DP, Andrews RE, Yousten AA. The genus *Bacillus* – insect pathogens. In: *The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, application*. 2nd ed. New York: Springer; 1992. p. 1697–745.
- Staswick PE, Serban B, Rowe M, Tiryaki I, Maldonado MT, Maldonado MC, et al. Characterization of an *Arabidopsis* enzyme family that conjugates amino acids to indole-3-acetic acid. *Plant Cell* 2005;17:616–27.
- Thilmony R, Underwood W, He SY. Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. tomato DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant J* 2006;46:34–53.
- Thomashow LS, Bonsall RF, Weller DM. Antibiotic production by soil and rhizosphere microbes *in situ*. In: *Manual of environmental microbiology*. 2nd ed. Washington, DC: ASM Press; 2002. p. 638–47.
- Thomma BPHJ, Tierens KFM, Penninckx IAMA, Mauch-Mani B, Broekaert WF, Cammue BPA. Different microorganisms differentially induce *Arabidopsis* disease response pathways. *Plant Physiol Biochem* 2001;39:673–80.
- Ticknor LO, Kolsto AB, Hill KK, Keim P, Laker MT, Tonks M, et al. Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. *Appl Environ Microbiol* 2001;67:4863–73.
- Toro M, Azcon R, Barea J-M. Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability and nutrient cycling. *Appl Environ Microbiol* 1997;63:4408–12.
- Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, et al. Acquired resistance in *Arabidopsis*. *Plant Cell* 1992;4:645–56.
- Vallad GE, Goodman RM. Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Sci* 2004;44:1920–34.
- van Loon LC. Systemic induced resistance. In: Slusarenko AJ, Fraser RSS, van Loon LC, editors. *Mechanisms of resistance to plant diseases*. Dordrecht: Kluwer; 2000. p. 521–74.
- van Loon LC, Glick BR. Increased plant fitness by rhizobacteria. In: Sandermann H, editor. *Molecular ecotoxicology of plants*. Berlin: Springer; 2004. p. 177–205.
- van Peer R, Schippers B. LPS of plant growth-promoting *Pseudomonas* sp. strain WCS417r induce resistance in carnation to *Fusarium* wilt. *Netherlands J Plant Pathol* 1992;98:129–39.
- Vargas-Ayala R, Rogriguez-kaban R, Morgan-Jones G, McInroy JA, Kloepper JW. Shifts in soil microflora induced by velvetbean (*Mucuna deeringiana*)

- in cropping systems to control root knot nematodes. *Biol Control* 2000;17:11–22.
- Walker TS, Bais HP, Grotewold E, Vitanco JM. Root exudation and rhizosphere biology. *Plant Physiol* 2003;132:44–51.
- Wei G, Kloepper JW, Tuzun S. Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. *Phytopathology* 1996;86:221–4.
- Weller DM, Raaijmakers JM, Gardener BBM, Thomashow LS. Microbial populations responsible for specific soil suppressiveness plant pathogens. *Annu Rev Phytopathol* 2004;40:309–48.
- Yan Z, Reddy MS, Kloepper JW. Survival and colonization of rhizobacteria in a tomato transplant system. *Can J Microbiol* 2003;49:383–9.
- Yan Z, Reddy MS, Ryu C-M, McInroy JA, Wilson M, Kloepper JW. Induced systemic resistance against tomato late blight elicited by plant growth-promoting rhizobacteria. *Phytopathology* 2002;92:1329–33.
- Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. *Soil Biol Biochem* 2002;34: 955–63.
- Zehnder G, Kloepper J, Yao C, Wei G. Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera: Chrysomelidae) by plant growth-promoting rhizobacteria. *J Econ Entomol* 1997;90: 391–6.
- Zehnder GW, Yao C, Murphy JF, Sikora EJ, Kloepper JW. Induction of resistance in tomato against cucumber mosaic cucumovirus by plant growth-promoting rhizobacteria. *Biol Control* 2000;45:127–37.
- Zhang S, Reddy MS, Kokalis-Burelle N, Wells LW, Nightengale SP, Kloepper JW. Lack of induced systemic resistance in peanut to late leaf spot disease by plant growth-promoting rhizobacteria and chemical elicitors. *Plant Dis* 2001;85:879–84.
- Zhang S, Moyne A-L, Reddy MS, Kloepper JW. The role of salicylic acid in induced systemic resistance elicited by plant growth-promoting rhizobacteria against blue mold of tobacco. *Biol Control* 2002;25:288–96.
- Zhang S, Reddy MS, Kloepper JW. Tobacco growth enhancement and blue mold disease protection by rhizobacteria: relationship between plant growth promotion and systemic disease protection by PGPR strain 90–166. *Plant Soil* 2004;262:277–88.
- Zhang W, Dick WA, Houtink HAJ. Compost-induced systemic acquired resistance in cucumber to *Pythium* root rot and anthracnose. *Phytopathology* 1996;86:1066–70.
- Zhang Z, Li Q, Li Z, Staswick PE, Wang M, Zhu Y, et al. Dual regulation role of GH3.5 in salicylic acid and auxin signaling during *Arabidopsis-Pseudomonas syringae* interaction^{1[W][OA]}. *Plant Physiol* 2007;145:450–64.
- Zheng XY, Sinclair JB. The effects of traits of *Bacillus megaterium* on seed and root colonization and their correlation with the suppression of *Rhizoctonia* root rot of soybean. *J Biocontrol* 2000;45:223–43.

Dynamic communication between plants and rhizobacteria via volatile signals

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Abstract

Certain plant growth-promoting rhizobacteria (PGPR), in the absence of physical contact with plants, stimulate plant growth and elicit induced systemic resistance (ISR) via volatile organic compound (VOC) emissions. Gas chromatographic analysis of VOCs collected from the PGPR strains *Bacillus subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a reveals consistent patterns in VOC emissions in comparison with non-growth promoting strains such as *E. coli* DH5 α . The two most abundant compounds, 2,3-butanediol and 3-hydroxy-2-butanone, are consistently emitted from GB03 and IN937a while these metabolites are not released from DH5 α . Transcriptional approaches have been employed to prove how *Arabidopsis* respond to biologically active bacterial VOCs. To assess potential utilization of PGPR VOCs for crop plants, volatile blends from GB03, IN937a, and DH5 α have been applied separately to pepper, tomato, and cucumber roots. Bacterial survival capacity of 2,3-butanediol null mutants was significantly reduced in proximity with plant tissue. These reduced bacterial survival rates suggest that in addition to bacterial VOCs triggering plant growth and induced systemic resistance in plants, such chemicals provide protection for

PGPR via chemical signaling within the host plant.

Rhizobacteria help plant growth and defense against multiple pathogens

Plant growth-promoting rhizobacteria (PGPR) include a wide range of root-colonizing bacteria with the capacity to enhance plant growth by increasing seed emergence, plant weight, and crop yields (Kloepper, 1980). Seed or seedling treatments with PGPR have been used to enhance growth of several crops (Glick, 1995) as well as to suppress the growth of plant pathogens and deleterious rhizosphere microorganisms. Proposed mechanisms for plant growth promotion by PGPR include bacterial synthesis of the plant hormones indole-3-acetic acid (IAA), cytokinin, and gibberellin; breakdown of plant-produced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase; and increased mineral and nitrogen availability (Glick et al., 1995; 1999).

In addition to promoting plant growth, application of certain PGPR strains to seeds or seedlings leads to a state of induced systemic resistance (ISR) in the treated plant (Kloepper et al., 1992). PGPR can influence pathogens by direct or indirect effects. Indirectly, root-colonization by certain rhizobacteria induces systemic resistance that is effective against viral, bacterial, and fungal pathogens, as well as nematodes (Kloepper et al., 1999). ISR occurs in many plants including carnation, cucumber, tobacco, tomato, bean, radish, and *Arabidopsis* (Van Loon et al., 1998; Ryu et al., 2003b; 2004b). In contrast to signaling pathways of necrotic pathogens or chemical-elicited systemic acquired resistance, ISR does not require a salicylic acid-dependent pathway (Van Loon et al., 1998; Ryu et al., 2003b; 2004b). Several microbial determinants have been associated with elicitation of ISR: 2,4-diacetylphloroglucinol (Iavicoli et al., 2003), the O-antigen from lipopolysaccharide (Van Peer and Schippers, 1992), and salicylic acid (Maurhofer et al., 1998).

Bacterial volatiles are as aromatic stimulants for plant growth and defense

By physically separating plant growth promotion rhizobacteria from their host plant airborne chemicals from certain soil bacteria have been identified as effective signals for triggering plant growth and ISR. (Ryu et al, 2003a; Ryu et al, 2004a). Of several PGPR tested, two strains, *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a, elicited plant growth promotion and ISR

by volatile organic compounds (VOCs) emissions. In the case of growth promotion by VOCs from strain GB03, the cytokinin receptor-deficient (*cre1*) and cytokinin- and ethylene-insensitive (*ein2*) mutants were insensitive to growth promotion effects triggered by GB03 volatiles, while ethylene-insensitive (*etr1*), auxin-transporter-deficient and ethylene-insensitive (*eir1*), gibberellic acid-insensitive (*gai2*), and brassinosteroid-insensitive (*cbb1*) mutants were all sensitive to GB03 VOCs as evidenced by measurement of growth promotion.

Of several *Arabidopsis* mutant lines tested for regulatory control of ISR against *Erwinia carotovora* subsp. *carotovora*, only the ethylene-insensitive line (*ein2*) did not exhibit an amelioration of disease symptoms when *Arabidopsis* plants were pre-treated with GB03 volatiles. To examine if ISR is mediated at the level of transcription, three transgenic β -glucuronidase (GUS) fusion lines involved in plant-defense signaling were tested. Of those lines assayed, the jasmonic acid and ethylene-responsive PDF 1.2 gene GUS fusion plant alone exhibited elevated GUS activity (>1000 fold) over untreated control plants (Ryu *et al.*, 2004).

Besides study of strain GB03 VOCs, characterization of the VOCs produced by IN937a, coupled with bioassays of fractions of VOCs elicited plant growth promotion allowed for an initial characterization of potentially active bacterial volatiles. Exposure of *Arabidopsis* to VOCs from strain IN937a resulted in a significant reduction in disease severity caused by *E. carotovora* subsp. *carotovora*. Elicitation of ISR occurred with exposure to bacterial VOCs for as little as 4 days. Various mutant lines of *Arabidopsis* were exposed to whole VOCs. Mutant lines included a jasmonic acid-insensitive line (*coi1*), an ethylene-insensitive line (*ein2*), a salicylic acid-degrading line (NahG), and a line that lacks a regulatory gene (*npr1*). VOCs from strain IN937a elicited ISR on all of these lines. Hence, elicitation of ISR by VOCs of IN937a is independent of jasmonic acid, ethylene, salicylic acid, and *npr1*. Such a pattern of signal pathway has not been reported with ISR elicited by bacteria, and therefore, it is likely that VOCs of IN937a elicit a distinct and uncharacterized pathway in *Arabidopsis*. The two most abundant compounds, 2,3-butanediol and 3-hydroxy-2-butanone (also referred to as acetoin), were consistently released from strains GB03 and IN937a while these metabolites were not released from DH5 α or water-treated MS media. Other components of the complex bacterial bouquet that did not elicit ISR included dodecane, 2-undecanone, 2-tridecanone, 2-tridecanol, and tetramethyl pyrazine.

In *Bacillus* sp., 2,3-butanediol and acetoin are produced with low atmospheric O₂ partial pressure to provide an alternative electron sink for the

regeneration of NAD^+ when aerobic respiration is limited possible. This additional metabolic pathway functions analogously to alcohol fermentation activated in yeast under anaerobic conditions. The biological activity of 2,3-butanediol in triggering ISR was surmised in *Arabidopsis* when pre-exposure of plants to low doses (pg to ng range) of 2,3-butanediol activated ISR. The priming activity of 2,3-butanediol to reduce a plant's susceptibility to disease was confirmed when Bacilli strains genetically blocked in the production of 2,3-butanediol exhibited no disease protection. More interestingly, direct application of bacterial volatiles into plant roots increased plant growth significantly and elicited ISR in the greenhouse (unpublished data).

Uncharacterized bacterial odors

The involvement of known signaling pathways in *Arabidopsis* were screened by exposing defined mutants and transgenic plant lines to bacterial emissions containing 2,3-butanediol. ISR triggered by strain GB03 VOC emissions was independent of salicylic acid, NPR1, and jasmonic acid signaling pathways, but did appear to be mediated by ethylene. Interestingly plant growth promotion activation by strain IN937a was independent of all the signaling pathways that were tested suggesting that additional VOCs utilize alternative pathways to trigger plant growth promotion (Ryu et al., 2003a). In fact, solid phase microextraction (SPME) analysis of bacterial odors turned up substantial differences in VOC profiles between two active strains GB03 and IN937a for growth promotion. Major branched-chain alcohols 3-methyl-1-butanol, 2-methyl-1-butanol and their conjugates were detected in IN937a, albeit not in GB03. Differences in volatile profiles might account for the dissimilarity observed among both strains in triggering growth promotion via volatile chemical signals, and suggests the existence of diverse VOC metabolism existing in two closely related Bacilli species (Frag et al., 2006).

Plant and bacteria responses to rhizobacterial volatiles

To understand plant-signaling pathways induced by bacterial VOCs during plant growth promotion and ISR, we have begun to characterize global changes in the *Arabidopsis* transcriptome using available microarray technology. Microarray results revealed physiological changes associated with growth, photosynthesis, and stress tolerance. In addition to bacterial synthesis of plant growth regulators such as auxin, bacterial VOCs induce auxin synthesis and transport *in planta*. Transcriptional analysis also

identified changes in cell wall loosening providing a possible mechanism for rapid cell expansion and leaf growth promotion that is observed in GB03 VOC exposure (Zhang et al 2007). 2,3-Butanediol also appears to participate in bacterial colization with its host.. In case of *Vibrio cholerae* the mutant defective in 2,3-butanediol synthesis exhibited reduced capacity to colonize the gut of its host the mouse and did not develop its usual biofilm. Preliminary observations with the 2,3-butanediol non-producing *Bacillus subtilis* strain also indicates poor colonization of its host, in this case, pepper roots and overall poor bacterial fitness *in situ*. It appears that 2,3-butanediol produced by *B. subtilis* may serve in a dual functions eliciting the production of plant antimicrobial compounds and as a protecting agent for bacterial cells (Yi et al., unpublished data). Additional investigations are underway to better characterize how PGPR utilize volatile signaling to transmit information between plants and rhizobacteria.

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Literature cited

- Cleyet-Marcel, J.C., Larcher, M., Bertrand, H., Rapior, S., and Pinochet, X. 2001. Plant growth enhancement by rhizobacteria. In: Nitrogen assimilation by plants, physiological, biochemical and molecular aspects. ed. Morot-Gaudry, J-F. Science Publishers, Inc. Enfield, NH, USA.
- Iavicoli, A., Boutet, E., Buchala, A., and Metraux, J.P. 2003. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Mol. Plant-Microbe Interact.* 16:851-858.
- Farag, M.A., Ryu, C.M., Sumner, L.W., and Paré P.W. 2006. Profiling of rhizobacterial emissions reveals prospective inducers of growth promotion and induced systemic resistance in plants. *Phytochemistry* 67:2262-2268.
- Glick, B.R. 1995. The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol.* 41:109-117.
- Glick, B.R. 1999. Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria, (eds). Glick BR, Patten CN, Holguin G, Penrose DM, Imperial College Press, London, UK.
- Kloepper, J.W., Leong, J., Teintze, M., and Schroth, M.N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* 286:885-886.
- Kloepper, J.W., Tuzun, S., and Kuc, J. 1992. Proposed definitions related to induced disease resistance. *Biocontrol Sci. Technol.* 2:349-351.

- Kloepper, J.W., Ryu, C.M, and. Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94:1259-1266.
- Kloepper, J.W., and Ryu, C.M. 2006. Bacterial endophytes as elicitors of induced systemic resistance. In: *Soil Biology, Vol.9 Microbial Root Endophytes*. B. Schulz, C. Boyle, and T.N. Sieber (eds.). Berlin Heidelberg: Springer-Verlag, Germany.
- Kloepper, J.W., Rodriguez-Kabana, R., Zehnder, G.W, Murphy, J., Sikora, E., and Fernandez, C. 1999. Plant root-bacterial interactions in biological control of soilborne diseases and potential extension to systemic and foliar diseases. *Austral. Plant. Pathol.* 28:27-33.
- Maurhofer, M., Reimmann, C., Schmidli-Sacherer, P., Heeb, S., Haas, D., and Defago, G. 1998. Salicylic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. *Phytopathology* 88:678-684.
- Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Wei, H.X., Pare, P.W., and Kloepper, J.W. 2003a. Bacterial volatiles promote growth in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA.* 100:4927-4932.
- Ryu, C.M., Hu, C.H., Reddy, M.S., and Kloepper, J.W. 2003b. Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. *New Phytol.* 160:413-420.
- Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Kloepper, J.W., and Pare, P.W. 2004a. Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol.* 134:1017-1026.
- Ryu, C.M., Murphy, J.F., Mysore, K.S., and Kloepper, J.W. 2004b. Plant growth-promoting rhizobacteria systemically protect *Arabidopsis thaliana* against Cucumber mosaic virus by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway. *Plant J.* 39:381-392.
- Van Loon, L.C., Bakker, P.A.H.M., and Pieterse, C.M.J. 1998. Systemic resistance induced by rhizosphere bacteria. *Ann. Rev. Phytopath.* 36:453-483.
- Van Peer, R., and Schippers, B. 1992. Lipopolysaccharides of plant-growth promoting *Pseudomonas* sp. strain WCS417r induce resistance in carnation to fusarium wilt. *Neth. J. Plant Pathol.* 98:129-139.
- Yoon, S.S., and Mekalanos, J.J. 2006. 2,3-Butanediol synthesis and the emergence of the *Vibrio cholerae* El Tor biotype. *Infect. Immun.* 74:6547-6556.
- Zhang, H., Kim, M.S., Krishnamachari, V., Payton, P., Sun, Y., Grimson, M., Farag, M.A., Ryu, C.M., Allen, R., Melo, I.S., and Pare, P.W. 2007. Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. *Planta* 226:839-51.

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Mixtures of Plant Growth-Promoting Rhizobacteria Enhance Biological Control of Multiple Cucumber Pathogens

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ABSTRACT

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Plant growth-promoting rhizobacteria (PGPR) strains INR7 (*Bacillus pumilus*), GB03 (*Bacillus subtilis*), and ME1 (*Curtobacterium flaccumfaciens*) were tested singly and in combinations for biological control against multiple cucumber pathogens. Investigations under greenhouse conditions were conducted with three cucumber pathogens—*Colletotrichum orbiculare* (causing anthracnose), *Pseudomonas syringae* pv. *lachrymans* (causing angular leaf spot), and *Erwinia tracheiphila* (causing cucurbit wilt disease)—inoculated singly and in all possible combinations. There was a general trend across all experiments toward greater suppression and enhanced consistency against multiple cucumber pathogens using strain

mixtures. The same three PGPR strains were evaluated as seed treatments in two field trials over two seasons, and two strains, IN26 (*Burkholderia gladioli*) and INR7 also were tested as foliar sprays in one of the trials. In the field trials, the efficacy of induced systemic resistance activity was determined against introduced cucumber pathogens naturally spread within plots through placement of infected plants into the field to provide the pathogen inoculum. PGPR-mediated disease suppression was observed against angular leaf spot in 1996 and against a mixed infection of angular leaf spot and anthracnose in 1997. The three-way mixture of PGPR strains (INR7 plus ME1 plus GB03) as a seed treatment showed intensive plant growth promotion and disease reduction to a level statistically equivalent to the synthetic elicitor Actigard applied as a spray.

Additional keywords: biocontrol, *Cucumis sativus*.

In response to environmental and health concerns about extended use of pesticides, there is considerable interest in finding alternative control approaches for use in integrated pest management strategies for crop diseases (36). It seems inevitable that fewer pesticides will be used in the future and that greater reliance will be placed on biological technologies including the use of microorganisms as antagonists (1,5). However, microorganisms as biological control agents typically have a relatively narrow spectrum of activity compared with synthetic pesticides (3,20) and often exhibit inconsistent performance in practical agriculture, resulting in limited commercial use of biocontrol approaches for suppression of plant pathogens (1).

Most approaches for biocontrol of plant diseases have used single biocontrol agents as antagonists to a single pathogen (47). This may partially account for the reported inconsistent performance by biocontrol preparations, because single biocontrol agents are not likely to be active in all soil environments in which they are applied or against all pathogens that attack the host plant. Control of a wide spectrum of pathogens by applied antagonists largely remains an unfulfilled goal for biological control. There are three main approaches to achieve this goal: (i) modify the genetics of the biocontrol agent to add mechanisms of disease suppression that are operable against more than one pathogen, (ii) alter the environment to favor the biological control agent and to disfavor competitive microflora, and (iii) develop strain mixtures with superior biocontrol activity (19). Several strategies for forming mixtures of biocontrol agents could be envisioned including mixtures of organisms with differential plant colonization patterns; mixtures of antagonists that control different pathogens; mixtures of antagonists with different mechanisms of disease suppression; mixtures of taxonom-

ically different organisms; or mixtures of antagonists with different optimum temperature, pH, or moisture conditions for plant colonization.

It is likely that most cases of naturally occurring biological control result from mixtures of antagonists, rather than from high populations of a single antagonist. For example, mixtures of antagonists are considered to account for protection with disease-suppressive soils (26,38). Consequently, application of a mixture of introduced biocontrol agents would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity, enhance the efficacy and reliability of control (12), and allow the combination of various mechanisms without the need for genetic engineering (19).

Previous studies on combinations of biological control agents for plant diseases have included mixtures of fungi (5,8-10,32), mixtures of fungi and bacteria (11,12,17,19,20,24-26,31), and mixtures of bacteria (10,21,22,28,33,34,37,39,41,42,44,46). Most of these reports on mixtures of biocontrol agents showed that combining antagonists resulted in improved biocontrol. However, there also are reports of combinations of biological control agents that do not result in improved suppression of disease compared with the separate antagonists (7,18,30,40). Incompatibility of the coinoculants can arise because biocontrol agents may also inhibit each other as well as the target pathogen or pathogens (24). Thus, an important prerequisite for successful development of strain mixtures appears to be the compatibility of the coinoculated microorganisms (2,10).

In most of the investigations conducted so far, the efficacy of individual biocontrol strains or strain combinations was assessed by screening these agents against a single isolate of the target pathogen. This could be another reason why selected biocontrol agents fail to be commercially successful, because often there is no consideration of the genetic diversity of the target pathogen (28). Little emphasis has been placed on identifying the potential effects of pathogen diversity on the efficacy of biological control agents. Significant genotypic variation in a fungal pathogen population may exist among geographic regions, within a given field, or even

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among lesions on the same plant (4,13,29). In addition, classical selection of biocontrol agents has focused on a single target pathogen and has not accounted for nontarget pathogens causing diseases on the host plants.

The overall goal of this investigation was to determine if improved efficacy and enhanced biological control consistency against multiple pathogens can be achieved by applying a combination of plant growth-promoting rhizobacteria (PGPR) strains, emphasizing strains that induce systemic resistance (35,45). The specific objectives were to test the potential of PGPR strain combinations for enhancing consistency and level of biocontrol against three pathogens and to evaluate the efficacy of PGPR mixtures in the field.

MATERIALS AND METHODS

Bacterial cultures. PGPR used in this study included *Bacillus pumilus* strain INR7, *Curtobacterium flaccumfaciens* strain ME1, *Burkholderia gladioli* strain IN26, and *Bacillus subtilis* strain GB03. Strain INR7 produced induced systemic resistance (ISR) in cucumber to anthracnose, angular leaf spot (44), and cucumber mosaic virus (CMV) (35), and strains ME1 and IN26 had ISR activity in nonpublished field trials with cucumber (G. Wei, *unpublished data*). Strain GB03, which has reported biological control activity against *Rhizoctonia solani* and *Fusarium* spp. (1) and which is part of a commercial product (Kodiak, Gustafson Inc., Dallas, TX), was provided as a wettable powder formulation by Gustafson Inc. For long-term storage, cultures were maintained at -80°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) that contained 20% glycerol. For PGPR seed treatment, vegetative cells grown on tryptic soy agar (TSA) for 24 h were suspended in 0.02 M phosphate buffer, pH 7.0, and an equal volume of 2% methylcellulose was then added. The bacterial suspension was mixed with cucumber seeds, resulting in 5×10^8 to 1×10^{10} CFU per seed, and then air-dried overnight in a laminar flow hood. Seed treatments with single strains resulted in population densities of 10^8 to 10^9 CFU per seed, and strain mixtures in which each strain was applied in equal amounts yielded 10^9 to 10^{10} CFU per seed. With all strain GB03 applications, a slurry of the wettable powder formulation Kodiak was prepared and then applied to cucumber seeds to yield mean densities of 5×10^9 CFU per seed.

Greenhouse study. A series of greenhouse experiments was conducted to assess control by PGPR against multiple pathogens, using the susceptible cucumber (*Cucumis sativus* L.) cultivar Straight 8. Treatments were arranged in a randomized complete block design with eight replications consisting of single plants per treatment. A total of nine treatments for each experiment were used for each study: three treatments consisting of single PGPR strains (INR7, ME1, and GB03); three treatments consisting of strain mixtures (INR7 plus ME1; INR7 plus GB03; and INR plus ME1 plus GB03); and three controls (a nonbacterized but pathogen-challenged disease; a nontreated healthy control; and a classical ISR control in which plant resistance was induced with the pathogen *Colletotrichum orbiculare* applied to the cotyledons 7 days prior to challenge).

Cucumber seeds were planted to a depth of approximately 1 cm in 10-cm² plastic pots (one seed per pot) containing 500 cm³ of soilless Pro-Mix growing medium (Premier Peat Ltd., Rivière-du-Loup, Quebec, Canada) and kept in the greenhouse with daily watering. Pathogens used for the challenge inoculation were *C. orbiculare*, causal agent of anthracnose disease, *Pseudomonas syringae* pv. *lachrymans*, causal agent of angular leaf spot, and *Erwinia tracheiphila*, causal agent of cucurbit wilt.

Inoculum of *C. orbiculare* was prepared by suspending spores from an 8-day-old culture grown on green bean agar (14) in sterile water to give a concentration of 10^5 spores per ml. Inoculum of *P. syringae* pv. *lachrymans* was prepared by suspending cells from a 24-h culture grown on TSA in distilled water to give a concentration of 10^9 CFU/ml. Inoculum of *E. tracheiphila* was prepared by grinding cucumber stem pieces infected with the cucumber wilt pathogen.

C. orbiculare and *P. syringae* pv. *lachrymans* were sprayed onto entire plants using a backpack CO₂ sprayer (model D-2015; R & D Sprayers, Opelousas, LA) and sprayed to run-off, and *E. tracheiphila* was injected (0.1 to 0.2 ml) with a syringe into the petiole of the first leaf.

A total of seven different experiments were conducted. Plants in each experiment were challenged with a single, double, or triple combination of pathogens 18 days after planting (DAP), and each experiment was repeated three times. All plants from experiments using *C. orbiculare* as the challenge pathogen were placed into a humidity chamber for 36 h immediately after inoculation. Seven days after pathogen challenge, the second and third leaf of each plant was assessed for anthracnose and angular leaf spot diseases, and the percent diseased leaf area of each was recorded. Cucurbit wilt was rated as the number of wilted leaves per total leaf number of each plant over an average of nine consecutive observation days; the disease progress was determined by measuring the area under the disease progress curve (AUDPC) as described by Tooley and Grau (43). Because of different amounts of disease in each experiment and heterogeneity of variances, data from three repeated trials were not combined, and one representative experiment from each series is presented. Data were analyzed with the general linear models procedure (GLM) in PC-SAS (SAS Institute, Cary, NC), and treatment means were separated using the least significance difference (LSD) test at $P = 0.05$.

Field trials. Two field trials were conducted at the E. V. Smith Horticulture Substation near Shorter, AL, with cucumber seeds from cultivar Straight 8 direct-seeded by hand-planting at 1-m spacing between plants in the field. In 1996, to evaluate control under two different disease pressures, a paired-plot design arranged in randomized complete blocks was used. One pair of each plot had source plants and the other did not; each pair consisted of a single row (replication) of eight plants. A total of eight treatments, each replicated six times with eight plants per replication, was used. Seed treatments consisted of the three PGPR strains INR7, ME1, and GB03, alone and in all possible combinations, plus a nonbacterized control. The same seed treatment procedure as described for the greenhouse study was used. Source plants were grown in the greenhouse without PGPR and were inoculated 1 week prior to transplanting to the field either with the two-leaf pathogens causing anthracnose and angular leaf spot or with the pathogen causing cucurbit wilt disease, and two of each were transplanted into experimental plots 21 DAP, after the plot was seeded. Disease on the direct-seeded cucumber plants (noninoculated plants) was monitored in both the plot pairs with and without inoculated source plants at 45 DAP by recording the number of leaves showing more than live lesions of angular leaf spot, which was the only disease in 1996. Marketable cucumber fruit were picked every 2 or 3 days until the end of the growing season, and cumulative weight was recorded.

In the second trial, conducted in 1997, the efficacy of PGPR treatments under high disease pressure created through source plants infected with anthracnose, angular leaf spot, and cucurbit wilt and then transplanted into experimental plots 21 DAP was investigated. The source plants were prepared as in 1996. The experiment was arranged in a randomized complete block design with eight treatments replicated six times and eight plants per replication. Treatments included strains INR7 and IN26 applied alone or in combination as a seed coat; spray application of each strain, INR7 and IN26, alone; a mixture of strains INR7, ME1, and GB03 applied as a seed coat; a positive control consisting of a spray application of the synthetic chemical Actigard (Novartis Crop Protection, Inc., Basel, Switzerland), the first commercial elicitor product with ISR activity; and a nontreated control. All spray applications were first applied 28 DAP and then again three more times at weekly intervals, each time spraying entire plants to run-off using rates of 10^8 CFU/ml for bacteria and 50 mg/liter for Actigard. Main runner length of cucumber plants was measured at 43 DAP, and a disease index was determined at 49 DAP according to a disease rating

TABLE 1. Disease protection induced by three plant growth-promoting rhizobacteria (PGPR) strains and their mixtures after single-pathogen challenge of cucumber plants under greenhouse conditions^a

Treatments ^w	<i>Colletotrichum orbiculare</i> ^x	<i>Pseudomonas syringae</i> pv. <i>lachrymans</i> ^x	<i>Erwinia tracheiphila</i> ^y
Disease control	75.9 a ^z	127.9 a	448.8 a
INR7	67.1 ab	84.1 bc	348.1 abc
ME1	33.0 c	70.8 c	337.1 abc
GB03	72.6 a	102.5 b	301.1 bcd
INR7 + ME1	45.9 abc	77.1 c	249.3 cd
INR7 + GB03	33.5 c	83.9 bc	270.0 cd
INR7 + ME1 + GB03	69.8 a	80.3 c	271.5 cd
ISR control	37.6 bc	12.3 d	396.3 ab
Healthy control	0.0 d	0.0 d	0.0 e
LSD	31.4	18.7	112.4

^a Experiments were repeated three times. One representative trial is presented for each pathogen.

^w PGPR strains applied as seed treatments are INR7 (*Bacillus pumilus*), ME1 (*Curtobacterium flaccumfaciens*), and GB03 (*B. subtilis*). Controls included a nonbacterized but pathogen-challenged disease control, a nontreated healthy control, and a classical induced systemic resistance (ISR) control, in which plant resistance was induced with the pathogen *C. orbiculare* on the cotyledons 7 days prior to pathogen challenge.

^x Values for *C. orbiculare* or *P. syringae* pv. *lachrymans* represent the sum of percent diseased leaf area of the second and third leaf of each cucumber plant from eight replications per treatment.

^y Values represent calculated area under disease progress curves of the number of wilted leaves per total leaf number of each plant from eight replications per treatment recorded over 7 consecutive observation days.

^z Means followed by different letters are significantly different according to the protected least significant difference (LSD) test at $P = 0.05$ using the general linear models procedure in PC-SAS

TABLE 2. Disease protection induced by three plant growth-promoting rhizobacteria (PGPR) strains and their mixtures after double-pathogen challenge of cucumber plants under greenhouse conditions^a

Treatments ^w	<i>Colletotrichum orbiculare</i> + <i>Pseudomonas syringae</i> pv. <i>lachrymans</i> ^w	<i>C. orbiculare</i> + <i>Erwinia tracheiphila</i> ^x	<i>E. tracheiphila</i> + <i>P. syringae</i> pv. <i>lachrymans</i> ^y
Disease control	170.9 a ^z	194.1 a	615.3 ab
INR7	141.0 b	192.4 ab	641.0 a
ME1	123.1 bc	186.5 ab	568.5 abc
GB03	133.9 bc	187.9 ab	499.6 abcd
INR7 + ME1	121.0 c	173.6 bc	428.1 cd
INR7 + GB03	126.4 bc	165.4 c	434.6 cd
INR7 + ME1 + GB03	131.3 bc	185.1 ab	385.0 d
ISR control	86.9 d	98.8 d	502.2 abcd
Healthy control	0.0 e	0.0 e	0.0 e
LSD ^z	19.5	19.2	157.3

^a Experiments were repeated three times. One representative trial is presented for each pathogen combination.

^w PGPR strains applied as seed treatments are INR7 (*Bacillus pumilus*), ME1 (*Curtobacterium flaccumfaciens*), and GB03 (*B. subtilis*). Controls included a nonbacterized but pathogen-challenged disease control, a nontreated healthy control, and a classical induced systemic resistance (ISR) control, in which plant resistance was induced with the pathogen *C. orbiculare* on the cotyledons 7 days prior to challenge.

^x Values for *C. orbiculare* or *P. syringae* pv. *lachrymans* represent the sum of percent diseased leaf area of the second and third leaf of each cucumber plant from eight replications per treatment.

^y Bacterial wilt did not develop because of the very severe expression of anthracnose; values represent the sum of percent diseased leaf area of the second and third leaf of each plant caused by *C. orbiculare*. Presented are the mean of eight replications of each treatment.

^z Bacterial leaf spot disease did not develop (less than 5% diseased leaf area); values represent calculated area under disease progress curves of the number of wilted leaves per total leaf number of each plant from eight replications per treatment over 9 consecutive observation days.

^z Means followed by different letters are significantly different according to the protected least significant difference (LSD) test at $P = 0.05$ using the general linear models procedure in PC-SAS.

scale from 0 to 5, in which 0 = no lesions, 1 = 1 to 20% of leaf area with lesions, 2 = 20 to 40% of leaf area with lesions, 3 = 40 to 60% of leaf area with lesions, 4 = 60 to 80% of leaf area with lesions, and 5 = 80 to 100% of leaf area with lesions. With this scale, the percentage of leaf area with lesions reflected the combined severity of anthracnose and angular leaf spot, both of which occurred in 1997. Data were analyzed using analysis of variance with GLM in PC-SAS, and treatment means were separated by the LSD test at $P = 0.05$ with PC-SAS.

RESULTS

Greenhouse study. The background level of disease obtained in the disease control treatment varied among the three trials of each experiment; however, the same trends for treatment effects on disease occurred in all three trials. Data in Tables 1 to 3 are from one representative trial of the three trials of each experiment. PGPR treatments induced significant disease protection compared with the noninduced disease control to varying degrees against *C. orbiculare*, *P. syringae* pv. *lachrymans*, and *E. tracheiphila* (Table 1). The frequency with which various PGPR treatments induced significant protection varied with the pathogen used. Against *C. orbiculare* and *E. tracheiphila*, one of the three tested PGPR strains applied individually induced significant reductions of symptoms compared with the disease control, while all three individual strain treatments significantly reduced symptoms of *P. syringae* pv. *lachrymans* (Table 1). The number of strain mixtures that induced significant protection was the same as the number of individual strains that significantly reduced symptoms of *C. orbiculare* and *P. syringae* pv. *lachrymans*, but was greater for *E. tracheiphila* (Table 1). The classical ISR control, in which plants were induced by inoculating the cotyledons with the pathogen *C. orbiculare* 7 days prior to challenge with the same pathogen, significantly reduced symptoms of *C. orbiculare* and *P. syringae* pv. *lachrymans*, but not *E. tracheiphila*.

TABLE 3. Disease protection induced by three plant growth-promoting rhizobacteria (PGPR) strains and their mixtures after triple-pathogen challenge of cucumber plants under greenhouse conditions^a

Treatments ^y	<i>Colletotrichum orbiculare</i> + <i>Pseudomonas syringae</i> pv. <i>lachrymans</i> + <i>Erwinia tracheiphila</i>	Diseased leaf area ^w	Live plants ^x
Disease control	93.1 a ^y	0.0	
INR7	72.5 b	37.5	
ME1	61.9 bc	0.0	
GB03	52.5 cd	0.0	
INR7 + ME1	54.8 cd	37.5	
INR7 + GB03	49.0 cd	25.0	
INR7 + ME1 + GB03	38.6 d	25.0	
ISR control	10.6 e	0.0	
Healthy control	0.0 e	100.0	
LSD	16.7	nd ^z	

^a Experiments were repeated three times. One representative trial is presented.

^y PGPR strains applied as seed treatments are INR7 (*Bacillus pumilus*), ME1 (*Curtobacterium flaccumfaciens*), and GB03 (*B. subtilis*). Controls included a nonbacterized but pathogen-challenged disease control, a nontreated healthy control, and a classical induced systemic resistance (ISR) control, in which plant resistance was induced with the pathogen *C. orbiculare* on the cotyledons 7 days prior to challenge.

^w Values represent the sum of percent diseased leaf area caused by *C. orbiculare* and *P. syringae* pv. *lachrymans* of the second and third leaf of each cucumber plant from eight replications per treatment.

^x Bacterial wilt was assessed as the percent of live plants 21 days after challenge, because typical progressive wilt symptoms did not develop after challenging with three pathogens.

^y Means followed by different letters are significantly different according to the protected least significant difference (LSD) test at $P = 0.05$ using the general linear models procedure in PC-SAS.

^z nd = not determined.

In the series of experiments in which plants were inoculated with two pathogens at the same time (Table 2), there was a trend to greater control with mixtures of strains. When the challenge pathogens were *C. orbiculare* and *P. syringae* pv. *lachrymans*, typical symptoms of both anthracnose and angular leaf spot resulted, and significant protection, relative to the noninduced disease control, was induced with all three individually applied PGPR as well as all strain mixtures. When challenged with both *C. orbiculare* and *E. tracheiphila*, anthracnose lesions developed within 5 to 6 days after pathogen inoculation. However, wilt symptoms were not observed and, therefore, results of only anthracnose symptoms were evaluated. With *P. syringae* pv. *lachrymans* and *E. tracheiphila* as the pathogens, angular leaf spot caused less than 5% mean diseased leaf area across all three repeated trials, so only wilt disease was measured as AUDPC values. In both these cases in which only one pathogen caused disease after a double-pathogen challenge (Table 2, columns 2 and 3), significant treatment effects were only detected with PGPR mixtures and not with individual strains.

In the three-way challenge of pathogens (Table 3), typical symptoms of anthracnose and angular leaf spot developed. Symptoms typical of cucurbit wilt (progressive wilting of whole plants starting with a single leaf at 10 to 14 days after inoculation) were not observed, and affected plants showed rapid total collapse and death at 18 to 20 days after inoculation, so wilt was evaluated by recording numbers of live or dead plants 21 days after challenge (Table 3). Results in Table 3, therefore, are given as two disease measurements; the first value represents the percentage of diseased leaf area caused by the two inoculated leaf pathogens rated 7 days after challenge, and the second value indicates the survival rate of plants 21 days after challenge. Significant reductions in diseased leaf area were induced by all three individual PGPR strains as well as all strain mixtures. The magnitude of protection was greater with strain mixtures than with individual strains. Reductions in symptoms of *E. tracheiphila* as measured by an increased percentage of live plants compared with the noninduced disease control were only detected with one of the three individual PGPR treatments, while all three strain mixtures induced reductions in symptoms. As in the experiments with single or double pathogen inoculations (Tables 1 and 2), induction with *C. orbiculare* did not lead to a reduction in symptoms of *E. tracheiphila*.

Overall, in seven different experiments repeated three times, mixtures consisting of two or three strains showed slightly greater levels of disease suppression as compared with single PGPR strains (Table 4). Means of disease reductions were 26.2% for mixtures and 21.7% for single strains. In addition, the consistency of obtaining significant disease suppression by PGPR treatments was increased with mixtures as compared with single strain treatments; mixtures induced significant disease sup-

pression in 12.3 trials, while single strains induced significant suppression in 8.7 of the 21 total trials.

Field trials. Although inocula of *C. orbiculare*, *P. syringae* pv. *lachrymans*, and *E. tracheiphila* were applied to source plants placed into challenged experimental plots, angular leaf spot was the predominant disease occurring in the 1996 field trial. All PGPR treatments significantly reduced the number of infected leaves with angular leaf spot in plots with and without the source plants (Table 5). The greatest level of disease suppression with and without source plants resulted with the treatment containing the three-strain mixture of PGPR. There were generally no statistically significant effects on yield in this trial, except for the combination INR7 plus ME1 under high disease pressure (Table 5).

In the field trial in 1997 (Table 6), all treatments led to significant increases in plant growth as measured by the length of the main runner 43 days after planting, but there were differences among

TABLE 5. Effects of individual plant growth-promoting rhizobacteria (PGPR) strains and strain mixtures on yield and severity of angular leaf spot disease at two inoculum potentials in the field in 1996^u

Treatment ^v	Natural field infestation		High inoculum potential by infected source plants ^w	
			Angular leaf spot ^x	Marketable yield (kg) ^y
Control	23.0 a ^z	13.5 a	43.7 a	13.8 b
INR7	7.1 bcd	15.7 a	16.5 bcd	13.9 b
ME1	12.3 b	16.2 a	20.8 bc	11.7 b
GB03	11.1 bc	16.0 a	19.8 bcd	15.7 ab
INR7 + ME1	4.6 cd	14.6 a	12.0 cd	18.0 a
INR7 + GB03	7.6 bcd	13.9 a	14.2 bcd	15.6 ab
ME1 + GB03	11.5 bc	17.1 a	25.0 b	15.1 ab
INR7 + ME1 + GB03	3.3 c	17.1 a	8.4 d	13.7 b
LSD	6.9	5.1	12.1	4.1

^u The field trial was conducted as a randomized complete block with two pairs of plots (with and without source plants), each with eight treatments, six replications per treatment, and eight plants per replication.

^v PGPR strains applied as a seed treatment are INR7 (*Bacillus pumilus*), ME1 (*Curtobacterium flaccumfaciens*), and GB03 (*B. subtilis*).

^w Source plants were infected in the greenhouse with either *Colletotrichum orbiculare* and *Pseudomonas syringae* pv. *lachrymans* or with *Erwinia tracheiphila* before two of each set of plants were placed into every paired experimental plot as 3-week-old transplants. Disease ratings are based on angular leaf spot, which was the predominant disease expressed in the field in 1996.

^x Number of leaves with more than five lesions per leaf were counted, and presented are means of six replications each with eight plants per treatment.

^y Cucumbers were picked twice a week as marketable fruits until the end of the season, and presented are cumulative mean yields per treatment.

^z Numbers with different letters are, showing significant differences at $P = 0.05$ according to the least significant difference (LSD) test procedure, using the general linear models procedure in PC-SAS.

TABLE 4. Summary of suppression of one, two, and three pathogens by individual plant growth-promoting rhizobacteria (PGPR) strains and strain mixtures over three repeated trials^v

Treatment ^w	Mean percentage of disease reduction (number of significant trials) ^x							Total Mean ^z	Total significant trials
	C ^y	P ^y	E ^y	C+P	C+E	P+E	C+P+E		
I + M + G	25.7 (1)	48.4 (3)	40.8 (3)	9.8 (1)	5.5 (1)	31.3 (1)	21.6 (1)	26.2	11
I + M	44.0 (2)	43.1 (2)	33.2 (2)	18.5 (2)	5.5 (1)	17.9 (1)	22.7 (2)	26.4	12
I + G	39.0 (2)	37.2 (2)	38.1 (3)	12.7 (1)	8.7 (2)	27.1 (2)	20.1 (2)	26.1	14
M	40.2 (2)	36.2 (2)	31.7 (1)	13.1 (1)	3.4 (0)	10.9 (0)	36.3 (3)	24.5	9
G	43.7 (2)	14.7 (2)	16.1 (1)	10.8 (2)	0.5 (0)	33.0 (1)	34.0 (2)	21.8	10
I	21.5 (0)	35.9 (2)	27.4 (1)	9.9 (1)	2.3 (0)	9.5 (0)	25.2 (3)	18.8	7
ISR control	68.4 (3)	69.8 (3)	18.4 (0)	59.8 (3)	43.7 (3)	-0.4 (0)	83.8 (3)	49.1	15

^v Summary of results from 21 greenhouse experiments (seven different pathogen challenges each with three trials).

^w I = INR7 (*Bacillus pumilus*), M = ME1 (*Curtobacterium flaccumfaciens*), and G = GB03 (*B. subtilis*) as a wettable powder formulation (Kodiak; Gustafson Inc., Dallas); induced systemic resistance (ISR) control = classical induction with the pathogen *Colletotrichum orbiculare* on the cotyledons 7 days prior to challenge.

^x Disease reduction and number of significant trials are based on comparisons to the noninduced, pathogen-inoculated control. Number of significant trials refers to the number of the three trials in which the disease reduction was statistically significant at $P = 0.05$. Mean percentage of disease reduction was calculated by averaging the percentage of disease reduction from each of the three trials.

^y C = *Colletotrichum orbiculare*, P = *Pseudomonas syringae* pv. *lachrymans*, and E = *Erwinia tracheiphila*.

^z Total mean represents the average (percent) disease reduction of treatments over all 21 conducted trials.

the treatments in the magnitude of growth promotion. The least significant growth promotion occurred with the Actigard treatment, which was significantly less than the growth promotion that resulted from treatment with strain INR7 as a seed treatment or foliar spray and from seed treatment with mixtures of strains (INR7 plus IN26; and INR7 plus ME1 plus GB03) (Table 6). Cucurbit wilt disease did not occur in 1997 due to the lack of the beetle vectors necessary to transmit the pathogen *E. tracheiphila* from the source plants. Consequently, disease assessments 49 DAP were based on the combined incidence of anthracnose and angular leaf spot using the 0 to 5 rating scale. All treatments led to significant disease suppression compared with the noninduced control, and there were significant differences in the magnitude of suppression among the PGPR treatments. Both strains IN26 and INR7 significantly suppressed disease when applied as seed treatments or foliar sprays, and with each application method, INR7 induced a significantly greater level of suppression than did IN26 (Table 6). The greatest magnitude of disease suppression occurred with Actigard, and the suppression resulting from seed treatment with the three-strain PGPR mixture was equivalent to the suppression from Actigard. The three-way PGPR strain mixture consisting of INR7, ME1, and GB03 provided control at a level statistically equivalent to the control by Actigard (Table 6). PGPR strain INR7 significantly reduced disease both as a seed treatment and as a spray application applied four times at weekly intervals.

DISCUSSION

Results presented here demonstrate that individual PGPR treatments induced significant disease protection against three pathogens inoculated individually and in two- or three-pathogen combinations and that mixtures of these PGPR strains exhibited a general trend toward greater disease suppression and more consistent attainment of significant disease suppression. In the summary of all 21 greenhouse trials (Table 4), treatments with the three single PGPR strains resulted in significant disease suppression as compared with the noninduced control in 7, 9, and 10 trials, while the three mixtures of PGPR led to significant protection in 11, 12, and 14 trials. Different mechanisms of action for different PGPR strains may explain why combinations of strains provided more consis-

tency in disease suppression. These results are in agreement with studies by Pierson and Weller (33) and Duffy and Weller (12), both of which demonstrated that certain mixtures of fluorescent pseudomonads were significantly more suppressive of take-all than either treatment used alone. Sung and Chung (42) demonstrated that chitinase-producing *Streptomyces* spp. and *Bacillus cereus* isolates used in conjunction with antibiotic-producing *P. fluorescens* and *Burkholderia* (*Pseudomonas*) *cepacia* isolates had a synergistic effect on the suppression of rice sheath blight. Because of the horizontal type of resistance associated with ISR (27), PGPR strains with ISR activity may increase the probability of achieving disease suppression against a wider range of pathogens compared with antagonists with antibiotic production as the main mechanism of biocontrol (23). Although the use of *C. orbiculare* (classical ISR control) proved to be a successful method to induce systemic resistance to anthracnose and angular leaf spot, the use of pathogens as inducers would not be practical agronomically because of the potential spread of disease. In addition, the classical ISR control failed to induce significant suppression of cucurbit wilt, and this occurred only with PGPR treatments.

The two field experiments in 1996 and 1997 investigating several different PGPR treatments demonstrated the feasibility of inducing resistance in cucumber plants under conditions of high inoculum potential (Tables 5 and 6). The comparison to Actigard indicated that some PGPR strains were as effective as this commercially available elicitor. The fact that a single application of PGPR strains as a seed treatment provided significant disease reduction through ISR in the field is particularly encouraging for the use of PGPR as components of integrated pest management systems. Other means of activating disease resistance (e.g., chemical inducers like Actigard applied as a spray) might require several applications, each of which would add costs for foliar spray application and would be dependent on favorable weather. Further, seed treatments provide targeted application of PGPR, allowing earlier protection than could be provided with foliar sprays. In addition, the additional plant growth promotion provided by some PGPR treatments in comparison to Actigard (Table 6) adds another advantage to PGPR over chemical inducers.

The still very limited number of compatible and effective mixtures of biocontrol agents is due to several factors. The majority of mixtures have no benefit or are detrimental to biocontrol activity. Further, a mixture that improves activity under one set of conditions or on one host may be antagonistic under another set of conditions or on a different host. From an economical point of view, a biocontrol product composed of a mixture of strains has a potential drawback, because producing and registering such a product will likely be more costly than a product composed of a single strain (39). However, greater emphasis on developing mixtures of biocontrol agents is needed, because they may result in better plant colonization, be better adapted to the environmental changes that occur throughout the growing season, present a larger number of pathogen-suppressive mechanisms, or protect against a broader range of pathogens (1).

Mixtures of biocontrol agents without chemical pesticides, as demonstrated in this study, will be of interest to the growing organic crop industry (in which, in order to be certified as organic, producers cannot use pesticides). For conventional agriculture, however, combinations of biological and chemical control seem to be the most promising area for the near future. Conway (6) reported that *Laetisaria arvalis* and a foliar spray of the experimental fungicide CGA 173506 at one-half the recommended rate reduced disease on rosemary more than treatment with either the biocontrol agent or fungicide alone. Commercial development has already been accomplished with two products marketed as Kodiak and Epic (Gustafson Inc.), in which two different *Bacillus subtilis* biocontrol strains were combined with a fungicide (carboxin-PCNB-metalaxyl) for use against soilborne diseases. During the 1996 season, approximately 5 million ha of crops were treated with these products, targeting

TABLE 6. Effects of individual plant growth-promoting rhizobacteria (PGPR) strains and strain mixtures on plant growth promotion and severity of anthracnose and angular leaf spot using artificially infected source plants in the field in 1997

Treatment ^w	Mean main runner length (cm) ^x	Anthrachnose and angular leaf spot (disease index) ^y
Control	44.8 d ^z	3.23 a
INR7	71.3 ab	1.68 cd
IN26	64.1 bc	2.39 b
INR7 + IN26	74.2 a	1.78 cd
INR7 + ME1 + GB03	72.0 ab	1.47 de
INR7 spray	72.2 ab	1.91 c
IN26 spray	60.0 bc	2.34 b
Actigard	61.6 c	1.24 e
LSD	8.98	0.33

^w Strains INR7 (*Bacillus pumilus*) and IN26 (*Burkholderia gladioli*) were coated onto cucumber seeds alone or in combination, or sprayed onto field plants four times at weekly intervals. Strains INR7, ME1 (*Curtobacterium flaccumfaciens*), and GB03 (*Bacillus subtilis*) were applied as a seed treatment mixture. The commercial elicitor product Actigard (Novartis Crop Protection, Inc., Basel, Switzerland) was used as a spray application four times at weekly intervals.

^x Mean main runner length in centimeters was measured 43 days after planting.

^y Disease assessment was done 49 days after planting the field by direct seeding according to a disease rating scale from 0 to 5 (0 = healthy to 5 = dead) based on the degree of disease infestation per plant.

^z Means followed by different letters are significantly different according to the protected least significant difference (LSD) test at *P* = 0.05 using the general linear models procedure in PC-SAS.

diseases of roots caused by *Rhizoctonia solani* and *Fusarium* spp., plus promoting increased root mass and plant vigor through hormone-like responses and disease control (1).

The results presented here indicate that mixtures of PGPR strains can enhance disease protection and improve the consistency of biological control. Therefore, it is essential to investigate microbial interactions that enhance or detract from biocontrol (16) to understand and predict the performance of mixtures of specific biocontrol agents.

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LITERATURE CITED

- Backman, P. A., Wilson, M., and Murphy, J. F. 1997. Bacteria for biological control of plant diseases. Pages 95-109 in: *Environmentally Safe Approaches to Crop Disease Control*. N. A. Rechcigl and J. E. Rechcigl, eds. CRC Lewis Publishers, Boca Raton, FL.
- Baker, R. 1990. An overview of current and future strategies and models for biological control. Pages 375-388 in: *Biological Control of Soilborne Plant Pathogens*. D. Hornby, ed. CAB International, Wallingford, United Kingdom.
- Baker, R. 1991. Diversity in biological control. *Crop Prot.* 10:85-94.
- Boeger, J. M., Chen, R. S., and McDonald, B. 1993. Gene flow between geographic populations of *Mycosphaerella graminicola* (anamorph *Septoria tritici*) detected with restriction fragment length polymorphism markers. *Phytopathology* 83:1148-1154.
- Budge, S. P., McQuilken, M. P., Fenlon, J. S., and Whipps, J. M. 1995. Use of *Coniothyrium minitans* and *Glucocladium virens* for biological control of *Sclerotinia sclerotiorum* in glasshouse lettuce. *Biol. Control* 5:513-522.
- Conway, K. E. 1997. Integration of biological and chemical controls for *Rhizoctonia* aerial blight and root rot of rosemary. *Plant Dis.* 81:795-798.
- Dandurand, L. M., and Knudsen, G. R. 1993. Influence of *Pseudomonas fluorescens* on hyphal growth and biocontrol activity of *Trichoderma harzianum* in the spermosphere and rhizosphere of pea. *Phytopathology* 83:265-270.
- Datnoff, L. E., Nemec, S., and Pernezny, K. 1995. Biological control of *Fusarium* crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*. *Biol. Control* 5:427-431.
- Datnoff, L. E., Nemec, S., and Pohronezny, K. 1993. Influence of *Trichoderma harzianum* and *Glomus intraradices* on incidence and severity of *Fusarium* crown and root rot. *Biol. Cultural Tests* 9:78.
- De Boer, M., van der Sluis, I., van Loon, L. C., and Bakker, P. A. H. M. 1997. In vitro compatibility between fluorescent *Pseudomonas* spp. strains can increase effectivity of *Fusarium* wilt control by combinations of these strains. Pages 380-382 in: *Plant Growth-Promoting Rhizobacteria-Present Status and Future Prospects*. Proc. Int. Workshop on Plant Growth-Promoting Rhizobacteria, 4th. A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo, and S. Akino, eds. Nakanishi Printing, Sapporo, Japan.
- Duffy, B. K., Simon, A., and Weller, D. M. 1996. Combination of *Trichoderma koningii* with fluorescent pseudomonads for control of take-all on wheat. *Phytopathology* 86:188-194.
- Duffy, B. K., and Weller, D. M. 1995. Use of *Gaeumannomyces graminis* var. *graminis* alone and in combination with fluorescent *Pseudomonas* spp. to suppress take-all of wheat. *Plant Dis.* 79:907-911.
- Fry, W. E., Goodwin, S. R., Matuszak, J. M., Spielman, L. J., and Milgroom, M. G. 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. *Annu. Rev. Phytopathol.* 30:107-129.
- Goode, M. J. 1958. Physiological specialization in *Colletotrichum lagenarium*. *Phytopathology* 48:79-83.
- Hall, R. 1995. Challenges and prospects of integrated pest management. Pages 1-21 in: *Novel Approaches to Integrated Pest Management*. R. Reuveni, ed. Lewis Publishers, CRC Press, Inc., Boca Raton, FL.
- Handelsman, J., and Stabb, E. V. 1996. Biocontrol of soilborne plant pathogens. *Plant Cell* 8:1855-1869.
- Hassan, D. G., Zargar, M., and Beigh, G. M. 1997. Biocontrol of *Fusarium* root rot in the common bean (*Phaseolus vulgaris* L.) by using symbiotic *Glomus mosseae* and *Rhizobium leguminosarum*. *Mol. Ecol.* 34:74-80.
- Hubbard, J. P., Harman, G. E., and Hadar, Y. 1983. Effect of soilborne *Pseudomonas* spp. on the biological control agent, *Trichoderma hamatum*, on pea seeds. *Phytopathology* 73:655-659.
- Janisiewicz, W. J. 1988. Biocontrol of postharvest diseases of apples with antagonist mixtures. *Phytopathology* 78:194-198.
- Janisiewicz, W. J. 1996. Ecological diversity, niche overlap, and coexistence of antagonists used in developing mixtures for biocontrol of postharvest diseases of apples. *Phytopathology* 86:473-479.
- Janisiewicz, W. J., and Bors, B. 1995. Development of a microbial community of bacterial and yeast antagonists to control wound-invading postharvest pathogens of fruits. *Appl. Environ. Microbiol.* 61:3261-3267.
- Johnson, K. B., Stockwell, V. O., McLaughlin, R. J., Sugar, D., Loper, J. E., and Roberts, R. G. 1993. Effect of antagonistic bacteria on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossoms and on fire blight control. *Phytopathology* 83:995-1002.
- Kloepper, J. W., Zehnder, G. W., Tuzun, S., Murphy, J. F., Wei, G., Yao, C., and Raupach, G. S. 1996. Toward agricultural implementation of PGPR-mediated induced systemic resistance against crop pests. Pages 165-174 in: *Advances in Biological Control of Plant Diseases*. T. Wenhua, R. J. Cook, and A. Rovira, eds. China Agricultural University Press, Beijing.
- Leeman, M., den Ouden, F. M., van Pelt, J. A., Cornelissen, C., Matamala-Garros, A., Bakker, P. A. H. M., and Schippers, B. 1996. Suppression of *Fusarium* wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. and root-colonizing fungi. *Eur. J. Plant Pathol.* 102:21-31.
- Leibinger, W., Beuker, B., Hahn, M., and Mendgen, K. 1997. Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. *Phytopathology* 87:1103-1110.
- Lemanceau, P., and Alabouvette, C. 1991. Biological control of *Fusarium* diseases by fluorescent *Pseudomonas* and nonpathogenic *Fusarium*. *Crop Prot.* 10:279-286.
- Lyon, G. D., and Newton, A. C. 1997. Do resistance elicitors offer new opportunities in integrated disease control strategies? *Plant Pathol.* 46:636-641.
- Mazzola, M., Fujimoto, D. K., Thomashow, L. S., and Cook, R. J. 1995. Variation in sensitivity of *Gaeumannomyces graminis* to antibiotics produced by fluorescent *Pseudomonas* spp. and effect on biological control of take-all of wheat. *Appl. Environ. Microbiol.* 61:2554-2559.
- McDonald, B. A., Miles, A. J., Nelson, L. R., and Pettway, R. E. 1994. Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum*. *Phytopathology* 84:250-255.
- Miller, R. H., and May, S. 1991. Legume inoculation: Successes and failures. Pages 123-134 in: *The Rhizosphere and Plant Growth*. D. L. Keister and P. B. Cregan, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.
- Park, C., Paulitz, T. C., and Baker, R. 1988. Biocontrol of *Fusarium* wilt of cucumber resulting from interactions between *Pseudomonas putida* and nonpathogenic isolates of *Fusarium oxysporum*. *Phytopathology* 78:190-194.
- Paulitz, T. C., Ahmad, J. S., and Baker, R. 1990. Integration of *Pyrhium nunn* and *Trichoderma harzianum* isolate T-95 for the biological control of Pythium damping-off of cucumber. *Plant Soil* 121:243-250.
- Pierson, E. A., and Weller, D. M. 1994. Use of mixtures of fluorescent pseudomonads to suppress take-all and improve the growth of wheat. *Phytopathology* 84:940-947.
- Raaijmakers, J. M., van der Sluis, I., Koster, M., Bakker, P. A. H. M., Weisbeek, P. J., and Schippers, B. 1995. Utilization of heterologous siderophores and rhizosphere competence of fluorescent *Pseudomonas* spp. *Can. J. Microbiol.* 41:126-135.
- Raupach, G. S., Liu, L., Murphy, J. F., Tuzun, S., and Kloepper, J. W. 1996. Induced systemic resistance in cucumber and tomato against cucumber mosaic cucumovirus using plant growth-promoting rhizobacteria (PGPR). *Plant Dis.* 80:891-894.
- Reuveni, R. 1995. *Novel Approaches to Integrated Pest Management*. Lewis Publishers, Boca Raton, FL.
- Roberts, D. P., Dery, F. D., Mao, W., and Hebrar, P. K. 1997. Use of a colonization-deficient strain of *Escherichia coli* in strain combinations for enhanced biocontrol of cucumber seedling diseases. *J. Phytopathol.* 145:461-463.
- Schippers, B. 1992. Prospects for management of natural suppressiveness to control soilborne pathogens. Pages 21-34 in: *Biological Control of Plant Diseases, Progress and Challenges for the Future*. NATO ASI Series A: Life Sciences. Vol. 230. E. C. Tiamos, G. C. Panavizas, and R. J. Cook, eds. Plenum Press, New York.
- Schisler, D. A., Slininger, P. J., and Bothast, R. J. 1997. Effects of antagonist cell concentration and two-strain mixtures on biological control of *Fusarium* dry rot of potatoes. *Phytopathology* 87:177-183.
- Sneh, B., Dupler, M., Elad, Y., and Baker, R. 1984. Chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* as affected by fluorescent and lytic bacteria from a *Fusarium*-suppressive soil. *Phytopathology* 74:1115-1124.
- Stockwell, V. O., Johnson, K. B., and Loper, J. E. 1996. Compatibility of bacterial antagonists of *Erwinia amylovora* with antibiotics used to control fire blight. *Phytopathology* 86:834-840.
- Sung, K. C., and Chung, Y. R. 1997. Enhanced suppression of rice sheath

- blight using combination of bacteria which produce chitinases or antibiotics. Pages 370-372 in: Plant Growth-Promoting Rhizobacteria-Present Status and Future Prospects. Proc. Int. Workshop on Plant Growth-Promoting Rhizobacteria, 4th. A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo, and S. Akino, eds. Nakanishi Printing, Sapporo, Japan.
43. Tooley, P. W., and Grau, C. R. 1984. Field characterization of rate-reducing resistance to *Phytophthora megasperma* f. sp. *glycinea* in soybean. *Phytopathology* 74:1201-1208.
 44. Waechter-Kristensen, B., Gertsson, U. E., Sundin, P., and Serra, G. 1994. Prospects for microbial stabilization in the hydroponic culture of tomato using circulating nutrient solution. *Acta Hort.* 361:382-387.
 45. Wei, G., Kloepper, J. W., and Tuzun, S. 1996. Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. *Phytopathology* 86:221-224.
 46. Wei, G., Kloepper, J. W., and Tuzun, S. 1996. Evaluation of induced systemic resistance and plant growth promotion with mixtures of plant growth-promoting rhizobacteria strains in the field. Pages 223-226 in: *Advances in Biological Control of Plant Diseases*. T. Wenhua, R. J. Cook, and A. Rovira, eds. China Agricultural University Press, Beijing.
 47. Wilson, M., and Backman, P. A. Biological control of plant pathogens. In: *Handbook of Pest Management*. J. R. Ruberson, ed. Marcel Dekker Publishers, New York. In press.